ASSOCIATION STUDY OF RECEPTOR GENES BETWEEN HEROIN ADDICTS AND CONTROLS

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TABLE OF CONTENT

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Abstract

Factors that contribute to drug addiction have been investigated extensively in recent years. In the past, environmental factors such as stress, peer group influence and drug availability were thought to be major contributors to addictive behaviour. More recently, through a number of twin and adoption studies, it was shown that genetic factors are also involved. It was shown that there was an 8-fold increased risk of drug disorders among the relatives of probands who abuse alcohol or other range of addictive substances like opioids and cocaine. Furthermore, a higher concordance rate for drug abuse was reported in monozygotic twins than in dizygotic twins. These findings encourage the exploration of genetic factors that cause addiction. As a result, most scientists have investigated the mesocorticolimbic pathway, which mainly consists of nucleus accumbens (NAc) and ventral tegmental area (VTA). These two regions of the brain may be the important part in the behavioral reward and reinforcement. In addiction, it was suggested that the dopaminergic, opioidergic and GABAergic systems participated in drug reward.

It was noted that the mu opioid receptor is the primary site of opiate action. Recent studies showed that the activation of mu opioid receptors in the VTA causes inhibition of GABA interneurons, in turn, it reduces the inhibition of the dopaminergic neurons. Ultimately, excitation of dopamine terminals is resulted. A number of association studies showed that there is an association between the *Taq*I A1 allele of D2 dopamine (DRD2) receptor gene, allele 9 of dopamine transporter (DAT) gene, gamma 2 subunit of GABA A (GABRG2) receptor gene and addictive behaviour like

alcoholism, cocaine dependence and gambling. Recently, it was shown that the A118G mutation site of the mu opioid receptor (MOR) gene and a polymorphism at the delta opioid receptor (DOR) gene may be associated with opioid dependence. Taken together, it is worth examining whether there is any association between heroin dependence and genes of the mesocorticolimbic pathway. This study involved using restriction fragment length polymorphism and denaturing HPLC (dHPLC) for genotyping DNA samples obtained from the heroin addicts in the Hong Kong Chinese population. In Hong Kong, approximately 87% of reported addicts are heroin abusers. An association was investigated involving the polymorphisms of the MOR exon 1 and intron 2, DOR exon 3, DRD2, DAT and GABRG2 genes.

In addition, the addiction severity index (ASI) was obtained for each heroin-dependent subject. The ASI examined the medical, employment, drug, legal, family/social relationships and psychiatry status of each subject, in order to assess the environmental factors that are associated with heroin dependence.

Results from the present study showed that the polymorphisms of A118G of exon 1 and C1031G of intron 2 of the human mu opioid receptor (hMOR) gene and the polymorphism of T921C of exon 3 of the human delta opioid receptor (hDOR) gene are significantly associated with heroin dependence. In addition, when an individual is found to carry a double dose of the variant gene, the vulnerability of heroin dependence is more robust as compared with those who carries a zero or single dose of the variant gene. Furthermore, the G allele of A118G and C1031G polymorphisms of the hMOR gene and the C allele of T921C polymorphism of the hDOR gene are

shown to favour heroin dependence.

The present results also showed that no significant association was found for the DRD2, DAT as well as the GABRG2 genes. Therefore, it maybe concluded that the polymorphism of mu and delta opioid receptor genes may contribute to the vulnerability of heroin dependence.

When the factors of the ASI were analyzed using the Pearson's correlation, a significant association was found in 3 (medical, employment and family/social relationships) out of 5 (medical, employment, drug, legal and family/social relationships) factors in the ASI. They were found to be correlated with psychiatric problems but not with drug problems. It seems therefore that environmental factors mainly affect the psychiatry status of the heroin-dependent subjects and not directly the nature of heroin dependence. Some of these factors may be related to their poor educational standards since 96.5% of subjects attained an educational standard no higher than Form 3, thus contributing to them having employment problems. The lack of employment may precipitate psychiatric symptoms like depression, anxiety and tension, which may also affect the subjects' family/social relationships. It is interesting to observe that the ASI drug status is only correlated to legal status, which may be due to the frequent police charges that these subjects received for possession of drugs or drug paraphernalia.

When genotype frequencies were correlated with the ASI, chi-square test showed statistical significant correlations between allelic frequencies and ASI factors between

G allele of the C1031G polymorphism and family/social relationships status as well as that of psychiatric status. Although the functional role of the hMOR and hDOR polymorphisms is still unclear, it may be possible that this polymorphism or other nearby mutation site which is in linkage disequilibrium with itself. Thus, this gene may play a role in the determination of personality or susceptibility to psychiatric disorders of these heroin-dependent subjects, which may in turn account for the poor relationships that they have with family members and in society.

LIST OF ABBREVIATIONS

APO	Apomorphine
ASI	Addiction severity index
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine 3',5'-monophosphate
CNS	Central nervous system
CRDA	Central registry of drug abuse
CREB	Cyclic AMP response element-binding protein
DA	Dopamine
DAT	Dopamine transporter
DOR	Delta opioid receptor
DRD2	D2 dopamine receptor
GABA	Gamma-aminobutyric acid
GABAA	Gamma-aminobutyric acid A
GABRG2	Gamma-aminobutyric acid receptor gamma 2 subunit
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
hDOR	Human delta opioid receptor
hKOR	Human kappa opioid receptor
hMOR	Human mu opioid receptor
hr	hour
ICSS	Intracranial self stimulation
K^+	Potassium ion

KOR	Kappa opioid receptor
min	minute
MOR	Mu opioid receptor
NAc	Nucleus accumbens
NF	Neurofilament
NS	Non-significant
PKA	Protein kinase A
PNS	Peripheral nervous system
PS-DVB	Poly-styrene divinylbenzyl
r	Pearson correlation
RFLP	Restriction fragment length polymorphism
RO	Relative odds
sec	second
SNPs	Single nucleotide polymorphisms
TEAA	Triethylammonium acetate
TH	Tyrosine hyroxylase
VNTR	Variable number of tandem repeats
VTA	Ventral tegmental area
χ^2	Chi-square test

實驗摘要

研究目的:近年,有很多關於導致毒品依賴因由的研究.從前,一般人相信環境因素例如壓力,同輩的影響和接觸毒品的容易度等都是引致一些可導致上癮的行爲的主因.但是,根據學生兒和收養兒童的研究指出.使人上癮的行爲可能與遺傳基因有關.若果家系中的先証者是有酷酒或吸食毒品的習慣.他/她的親屬及親戚會比較其他沒有酷酒或吸毒的先証者的家庭成員有高出八倍的機會染上酒,毒癮.在人腦中,中腦皮質邊緣葉裡的兩個主要部份:中腦被蓋和伏隔核會是可使上癮的行爲的介質.所以在這些部份可找到的多巴胺能的系統,阿片系統和加馬氨基丁酸系統可能與毒品依賴有關.

方法:這個實驗主要是研究以下幾個基因:μ阿片受體基因的A118G和C1031G多型性,及δ阿片受體基因的T921C多型性,D2多巴胺基因的*Taq*IA多型性,多巴胺運輸體基因的多型性和加馬氨基丁酸A受體加馬亞單位的*Nci*I多型性.從而找出這群基因的多型性會否與海洛英依賴有關連.6毫升的血液會從97位對照者和200位海洛英依賴者抽取.從中提取的脫氧核糖核酸是用作分析基因型.另外,一份問卷名爲癮癖嚴重指數則用作收集參與者的健康狀況,工作,藥物濫用,刑事紀錄,人際關係以及精神狀態方面的資料.再找出這些環境因素與海洛英依賴有否直接關係.

實驗結果:從癮癖嚴重指數的資料顯示,海洛英依賴與健康狀況,工作,人際關係以及精神狀態沒有直接關聯.從基因型分析結果,只有μ阿片受體基因的A118G和C1031G多型性,及δ阿片受體基因的T921C多型性與海洛英依賴有關.加上,當一個人擁有兩劑特變基因時, 吸食海洛英的傾向性便更加明顯.

總結:基因的多型性可能會是導致不同人對海洛英依賴的不同反應程度.A118G多型性的特變基因G等位基因,C1031G多型性的特變基因G等位基因以及T921C多型性的特變基因C等位基因可能是會導致海洛英依賴的基因.

CHAPTER ONE INTRODUCTION

1.1 Heroin

Heroin (O^3, O^6-diacetylmorphine) is a common Schedule I semisynthetic drug. Street names associated with heroin include "smack", "H", "horse" and "junk". Heroin was named because of its heroic property (Pinger et al., 1998). Pure heroin is a white, crystalline, soluble powder with a bitter taste. A bag of 100 mg of heroin sold on the street is not pure. It is diluted with lactose, mannitol, dextrose, talc, milk powder or quinine at each level of distribution (Schwartz, 1998). Most illicit heroin varies in colour from white to dark brown because of impurities.

Another form of heroin, known as "black tar", has also become readily available in Central American countries like Mexico and Guatemala. This dark Mexican heroin usually ranges from a dark brown powder to a black tar, which is the result of the crude processing methods used to illicitly manufacture heroin in a faster and simpler way (Bono, 1998).

There are 3 common self-administration patterns of heroin. It can be smoked, snorted or injected. The latter is the most efficient way to take this drug. However, the most popular way of heroin administration in Hong Kong is the practice popularly referred to as "chasing the dragon" (Karch, 1996). The heroin powder is heated on a piece of tin foil over a flame and the user inhales the fume through a straw. The heroin is dyed red and the upwardly curling fume resembles the tail of a dragon (Jenkins et al.,

1994).

1.1.1 Historical Background

Opium is said to have been introduced to China as a medicinal trade item by the Arabs during the eighth-century T'ang dynasty, or perhaps a century before. Chinese edicts not only oppose the importation of opium but also limited all trade and this had been proliferating since the early eighteenth century. However this did not stop traders from evading the laws by bribery, by shipping to other ports or by smuggling. To suppress the opium trade, the emperor appointed Lin Tse-hau, Commissioner Lin, and gave him broad powers. In March 1839, Lin arrived in Canton and demanded the surrender of all the opium in the traders' possession as well as a guarantee that they would stop shipping opium. Unfortunately, this did not happen and continued ship seizures along with misunderstandings eventually led to the Opium Wars. Traders who refused to cease shipping opium to China provoked the first Opium War between China, Britain and other nations. The war lasted from 1839 until the signing of the Treaty of Nanking in 1842. By 1856, with piracy, opium smuggling and addiction at an all-time high, the Chinese were again desperate to end the trade. The Second Opium War declared by the British ended with another defeat for China. In 1858, the Treaty of Tientsin gave the foreigners even more freedom and power in opium trade. The West discovered that opium smoking could not be contained in distant colonies and that it had spread to Europe and North America, lending another facet to the world's growing drug problem (Hodgson, 1999).

In 1847, C.R Wright in London first synthesized heroin, one of the derivatives of opium. This process involved the boiling of anhydrous morphine with acetic anhydride and produced a series of acetylated morphine derivatives. One of the derivatives was diacetylmorphine (heroin) (Eddy, 1953). In 1898, Strube reported that heroin could be used in treatment for patients with tuberculosis and for relief of severe coughs without ill effects (Strube, 1898). In the same year in Germany, scientists at the Bayer Company, known for manufacturing aspirin, concluded that heroin produced less respiratory depression than codeine. Based on these findings, the Bayer Company started producing heroin commercially as a safer cough suppressant. Originally, heroin was considered a better analgesic than morphine, a more efficient cough suppressant and a non-addictive cure for morphine and opium withdrawal symptoms. However, 12 years later, it was revealed that heroin was at least as addictive as morphine (de Ridder, 1994).

Since the establishment of the United States Pure Food and Drug Act of 1906, an accurate label is required on all drugs sold interstate, specifically referring to heroin, morphine, opium and cocaine (Pinger et al., 1998). In the 1920s, because of its higher potency and illicit availability, heroin addiction became a serious problem (Richard, 1974). In view of this, the Narcotic Drug Import and Export Act of 1924 was amended in the United States to prohibit the importation of opium for the manufacturing of heroin. Meanwhile, heroin became available in the black market. One year later, the International Opium Convention limited the supply of heroin from Europe. Shortly thereafter, it was found that heroin was manufactured secretly in China. (Bono,1998). Heroin abuse accelerated gradually in the 1960s when other

drugs, such as hallucinogens, became popular. In 1970, the Controlled Substances Act classified heroin as a Schedule I drug. This means that heroin is a drug susceptible to abuse and without any medical value at all (Richard, 1974).

During most of the 1980s, the supply of heroin in the United States mainly came from Southwest Asia, Pakistan, Iran, Afghanistan and Lebanon. In the early 1990s, the high-purity Southwest Asian heroin dominated the United States heroin market especially on the East Coast of the United States. The primary sources of that heroin are the Golden Triangle countries of Thailand, Laos and Burma, the Golden Crescent countries of Iran, Afghanistan and Pakistan, Mexico and most recently Columbia (Pinger et al., 1998). Traditionally, the purity of heroin ranged from 1 to 10%, more recently heroin purity can reach 98%. Since 1990, the national average purity of heroin sold on the streets increased dramatically from 24 to 35.8% in 1993. Heroin with the highest purity came from South America, averaging 59.3%, followed by 47.2, 32.2 and 27.8% from Southwest Asia, Southeast Asia and Mexico respectively. In 1995, the estimated world production of opium was 3400 metric tons. More than half was produced in Southeast Asia (Thailand, Laos and Burma), 1100 tons in Southwest Asia (Afghanistan and Pakistan) and 60 tons in South America and Mexico (Karch, 1996).

In Hong Kong, according to the Hong Kong Government Central Registry of Drug Abuse (CRDA) report, it was noted that throughout the past decade, heroin continued to be the most popular drug of abuse in Hong Kong. Of the 13,297 individuals reported in 1989 who are substance abusers, 92.9% of those were reported to abuse

heroin. The percentage of heroin abusers among the reported addicts fluctuates between 92% and 94% from 1989 to 1994 (CRDA report 1989-1994, 1995). However, it was indicated that there is a decline in the predominance of heroin abuse in Hong Kong as other psychotropic substances such as amphetamine, cannabis, triazolam and cough medicine are emerging gradually. The percentage of reported heroin abusers dropped from 89.0 % in 1995 to 86.6% in 1999.

1.1.2 Manufacturing of Heroin

By chemically altering morphine that was derived from the opium poppy, heroin can be manufactured. Among the 6 genera in the Papaveraceae family, Papaver somniferum was commonly cultivated as the "opium" poppy. The poppy is an annual plant. Although it grows in almost any climate, the optimal condition for its cultivation is warm and temperate.

Opium poppies show variations in height, colour, shape and the number of capsules on each plant. After the flowers have fallen off, the ripened capsule is cut, allowing the latex to run down inside the plant. It takes 8-14 hours for the latex to become solidified; by using a dull blade, the latex is scraped off the capsule. Morphine is the principal alkaloid found in crude opium (Anon, 1963). Codeine and thebaine are also significant alkaloids present in opium. Opium contains approximately 10 to 12% morphine and 0.5 to 1.0% codeine as well as many other alkaloids (Yagiela, 1998).

1.1.3 Route of Administration and Absorption Rate

Heroin can be self-administered by ingestion (placed under the tongue), inhalation such as smoking or snorting, as well as by intravenous, intramuscular or subcutaneous injection.

Oral administration is a familiar way to introduce heroin into the body. After heroin is dissolved in the stomach, it is then carried to the intestine and absorbed through the intestinal mucosa by passive diffusion. However, the relative degree of contact with the mucosa determines the amount of uptake in each segment. Although it is a convenient and safe route and the orally taken heroin does not have to be sterilized, the abusers who use this route may experience occasional nausea and stomach distress (Yagiela, 1998). The absorption of an oral dose of heroin is poor and incomplete, since acids in the stomach may break it down, or because it binds to other contents in the digestive tract or is excreted in the faeces. Therefore, it is difficult to estimate the oral dosage of heroin (Pinger et al., 1998).

Inhalation is an increasing popular method of drug administration, although snorting heroin is the usual way for those who begin heroin use. Heroin that is inhaled reaches the mucous lining of the nose and throat before being absorbed into the bloodstream and reaches the alveoli of the lungs easily and rapidly. This method avoids the unpredictability of administration through the gastrointestinal tract. Furthermore, it eliminates the pain and discomfort that is associated with the injection route. However, inhalation may lead to irritation to the throat and lungs or deterioration of

their cell membranes (Pinger et al., 1998).

Smoking of heroin has been a popular route of administration in Eastern culture. This practice is named " Chasing the Dragon". Alternatively, the heroin powder can be smoked through the lighted end of a cigarette. Smokers usually tilt their head backwards to prevent the heroin from falling off the cigarette (Karch, 1996).

Another administration pattern is the injection of heroin. This can be done intravenously, intramuscularly or subcutaneously. However, since the 1980s, most users prefer the intravenous route in which the injected heroin powder bypasses the body's defense layers (skin, mucous membrane, lining of the alimentary canal) and then reaches the bloodstream very rapidly (Pinger et al., 1998). Heroin powder is prepared in a spoon and was diluted in a little water. Then the powder is boiled over a match, candle or cigarette lighter for a few seconds. In turn, a piece of cotton is used to filter out the impurities before injection. Then, the user looks for the large vein in the arm to inject the heroin and the "rush" follows immediately (Richard, 1974). The peak concentration in blood is achieved within 1 to 5 minutes after intravenous injection (Jenkins et al., 1994), and 5 minutes after intranasal and intramuscular injection (Cone et al., 1993). However, this practice has several drawbacks. There is an increase risk of infection and transmission of a variety of diseases such as HIV and hepatitis B virus resulting from the use of unsterile needles (Pinger et al., 1998).

1.1.4 Metabolism of Heroin

The lipophilic characteristics of heroin promote its passage through biological membranes like the blood brain barrier and subsequent access to the site of action at the brain's opioid receptors (Nutt, 1996). However, the biotransformation of heroin into its more hydrophilic metabolites is essential for the termination of its biological activity and the elimination of these compounds from the body. The metabolic conversion of heroin is an enzymatic reaction. In the liver, the cytochrome P450 enzyme family localized in the microsomes of the endoplasmic reticulum is involved. Other organs with significant metabolic capacity including the kidneys, gastrointestinal tract, skin and lungs may also participate. Normally, a significant portion of a dose of heroin may be metabolized in either the liver or intestines before it reaches the bloodstream (Pratt and Taylor, 1990).

The biotransformation reactions are classified as Phase I functionalization reactions and Phase II biosynthetic reaction. During Phase I, heroin is converted rapidly to biologically active metabolites, like 6-monoacetylmorphine and morphine, by the hydrolysis of ester or amide linkages (Benet et al., 1996). As a result, after morphine is formed, it will then be subjected to N-dealkylation. (Fischman and Hahn, 1978). Subsequently, normorphine is produced which to in turn enters Phase II, conjugation reactions. As a result, the major metabolite of heroin, the 3-glucuronide of morphine and the minor quantities of morphine-6-glucuronide are formed (Benet et al., 1996).

Following intravenous infusion of 70mg of heroin to human volunteers, 45% of the

dose was found in urine 40 hours (h) later. This consists of 38% conjugated morphine, approximately 4% free morphine, 1% 6-monoacteylmorphine and 0.1% diacetylmorphine (Baselt and Cravey, 1995). Urinary elimination half-lives of 0.6, 4.4 and 7.9 h were reported for 6-monoacteylmorphine, morphine and conjugated morphine respectively after administration of 6mg of heroin by the intramuscular route (Cone et al., 1991).

1.1.5 Physical and Psychological Effects of Heroin

Administration of heroin produces analgesia, euphoria, and drowsiness. However, heroin does not cause the slurring of speech to the degree that alcohol and barbiturates do. Moreover, heroin reduces the sensitivity of the brainstem to levels of carbon dioxide that may be building up in the body. When a higher dose is administered, breathing becomes irregular and slow (MMWR, 1989). Other undesirable effects include constipation, stimulation of vomiting and suppression of rapid eye movement (REM) sleep (Pinger et al., 1995).

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Tolerance develops when the dosage of administration has to be increased in order to obtain the same effects as the initial dose. Symptoms of physical dependence on heroin appear with the abrupt termination of chronic heroin administration. The symptoms that appear after a period of abstinence are referred to as withdrawal syndromes. They include anxiety, restlessness, irritability, general body aches, insomnia, runny nose and eyes, perspiration, fever and chills, hot flushes, dilated pupils, nausea, gagging, vomiting, diarrhea, increased heart rate, increased blood

pressure and abdominal and other muscle cramps (Platt, 1986; Pinger et al., 1995).

It must however be noted that the psychological effects of heroin vary with individuals. The acute effects of heroin use can be observed outwardly as a behavioral pattern of lethargy, lack of concern and inability to concentrate. Inwardly, abusers feel peaceful together with experiencing feelings of well-being and euphoria. In addition, for intravenous users, during the rush, sexual pleasures can be experienced. Nevertheless, these highs last only a short time and are followed by a period of increased anxiety. Because tolerance develops, evidence of craving can be observed. The abusers as a result will self-administer continual doses without the need for other natural rewards like food and water in order to enjoy the positive effects and avoid the aversive ones (Pinger et al., 1995). The emotions experienced range from mild feelings of pleasure or relief of tension through stronger emotional drives that can lead to persistent use, to changes in lifestyle and personal commitments, including keeping the company of those who are similarly involved with the drug (Pinger et al., 1998).

1.2 Opioid Receptors

Opioid receptors are widely distributed throughout the central nervous system (CNS) and the peripheral nervous system (PNS). Several types of opioid receptors exist, they are mu, delta, kappa and sigma. Each has an unique pharmacological profile, anatomical distribution and function (Van Ree et al., 1999). However, only the mu, delta and kappa opioid receptors have been cloned and widely studied. In the 1990s,

opioid receptors were isolated, purified, cloned, sequenced, and the three-dimensional structures modeled, first in laboratory animals and then in human beings (Thompson et al., 1993; Knapp et al., 1995; Minami and Satoh, 1995). All opioid receptors belong to a superfamily of guanine nucleotide regulatory proteins (G-protein)-coupled receptors, all of which possess seven membrane-spanning regions. All opioid receptors mediate their effects via the closely linked G-proteins as well as a variety of second-messenger systems such as adenylate cyclase, potassium (K^+) and calcium (Ca^{2+}) channels, and phospholipase C (Homanics et al., 1998). Each opioid receptor type is coded for by a distinct gene and is expressed through a specific messenger RNA (Atcheson and Lambert, 1994). The enkephalins (Hughes et al., 1975), dynorphins (Goldstein et al., 1979) and beta-endorphin (β-endorphin) (Bradbury et al., 1975) were identified as endogenous peptide ligands for the opioid receptors. Both enkephalins and dynorphins are neurotransmitters in the brain involved in pain preception, cognitive functions, affective behaviours and locomotion (Simon, 1991). Delta opioid receptors (DOR) exhibit low affinity for dynorphin, but are highly sensitivity to enkephalin. Dynorphin A is very potent at kappa opioid receptors (KOR), but these receptors have low affinity for enkephalin. Mu opioid receptors (MOR) exhibit high affinity for enkephalin, whereas β-endorphin binds potently to both DOR and MOR but has relatively lower affinity at KOR (Delfs et al., 1994).

1.2.1 Mu Opioid Receptors (MOR)

MOR is the primary site for the action of opioids including morphine, heroin, fentanyl and methadone. It has been classified into two subtypes, mu 1 and mu 2 according to

the relatively high and low binding affinity for opiate agonists such as naloxonazine and morphine respectively (Pasternak and Wood, 1986). In 1993, Chen and colleagues cloned cDNAs of the MOR from the rat brain. It was demonstrated that MOR possess a putative secondary structure of 7 transmembrane domains common to G-protein coupled receptors and display functional coupling to adenylyl cyclase (Chen et al., 1993). Subsequently, Wang et al. isolated an 18-kb genomic clone and showed by in situ hybridization that the human MOR gene (hMOR) mapped to chromosome 6q24-q25 (Wang et al., 1994).

MOR are present in both the brain and spinal cord. These receptors are widely distributed in the PNS and CNS. They are located in the superficial spinal cord, trigeminal nucleus caudalis, brainstem nuclei such as the chemoreceptor trigger zone, nucleus of the solitary tract, respiratory nuclei, cough center and related areas. They are also found in periaqueductal gray and periventricular zones of the midbrain, striatum, thalamus, amygdala, locus coeruleus neurons and nucleus accumbens (NAc). NAc is the region where the compulsive abuse of opioids, stimulants or drugs subject to compulsive abuse is implicated. However, there are few or no MOR in the cerebral cortex and cerebellum.

The activation of MOR produce an inhibition of adenylate cyclase and a reduction of the level of cyclic adenosine 3',5'-monophosphate (cAMP) (Murthy and Makhlouf, 1996). According to electrophysiological studies, its activation also opens K^+ channels resulting in the hyperpolarization of gamma-aminobutyric acid (GABA) interneurons. As a result, the dopaminergic neurons are released from the inhibition

by the GABA interneurons and in turn, the firing of dopaminergic neuron is increased and an increased release of DA resultes (Johnson and North, 1992). Studies on mu-receptor knockout mice suggest that MOR may play a role in analgesia (Matthes et al., 1996; Sora et al., 1997), respiratory depression (Shook et al., 1990), meiosis, gastrointestinal motility (Kromer, 1988), euphoria, reward, withdrawal (Homanics et al., 1998) and immunosuppression (Bryant and Holaday, 1993).

From genetic studies, five single-nucleotide polymorphisms (SNPs) in the coding region of the hMOR exon 1 have been identified (Wang et al. 1994; Mestek et al. 1995; Bergen et al., 1997; Berrettini 1997; Bond et al., 1998). The most prevalent SNP is a nucleotide substitution at position 118 (118A-G) of the coding region, predicting an amino acid change at a putative N-glycosylation site. The 118 A-G (A118G) variant receptor was found to bind β-endorphin approximately 3 times more tightly than the most common allelic form of the receptor. Furthermore, β-endorphin is approximately 3 times more potent at the A118G variant receptor. Therefore, the A118G polymorphism in hMOR gene may implicate a variation of vulnerability to develop addiction due to the different sensitivity to opioids (Bond et al., 1998). Smolka et al (1999) tested whether the A118G polymorphism is associated with a variation in central dopaminergic sensitivity during alcohol withdrawal in alcoholics. This study suggested that the A118G polymorphism modulates the central dopaminergic sensitivity during acute alcohol withdrawal. The apomorphine (APO)-induced growth hormone (GH) response represents an indicator of central dopaminergic sensitivity. Results showed that there was a 2-fold higher APO-induced GH response one week after alcohol cessation in alcoholics carrying the A118G

variant as compared with those alcoholics with the wild-type genotype (Smolka et al., 1999). It was postulated that the change in dopaminergic activity may also contribute to opioid dependence.

A C17T variant was also found in the coding region of the hMOR gene exon 1. The C17T polymorphism changes an alanine residue to a valine residue. Although the C17T variant was present in higher proportion among 55 opioid-dependent subjects than the 51 controls, it was only at a marginal significance level (p=0.054) (Berrettini et al., 1997). In view of this result, it was suggested that further studies on polymorphisms at the hMOR genes are necessary for the investigation of association between opioid dependence and the hMOR gene.

1.2.2 Kappa Opioid Receptors (KOR)

High concentrations of KOR are found in the basal ganglia, NAc, ventral tegmental area (VTA), deep layers of the cerebral cortex, hypothalamus, periaqueductal gray and dorsal horn of the spinal cord. In 1995, Satoh and Minami hypothesized that KOR is synthesized and transported to the terminals of dopaminergic neurons and plays a role in reducing the release of dopamine (Minami and Satoh, 1995). Yasuda and coworkers mapped the human KOR (hKOR) gene to chromosome 8q11.2 by fluorescence in situ hybridization (Yasuda et al., 1994). The KOR resemble the MOR since they both possess a putative secondary structure of 7 transmembrane domains common to G protein-coupled receptors and affect the activity of the adenylyl cyclase. The activation of the KOR reduces Ca^{2+} ion currents and increases K^{+} ion

conductance. Subsequently, there is an inhibition of neurotransmitter release. KOR are involved in analgesia (Pasternak, 1993), dysphoria, miosis (Dalman and O'Malley, 1999), some psychotomimetic effects such as disorientation and depersonalization feelings, and mild respiratory depression (Kumor et al., 1986). Although the function of KOR remains unclear, recent research indicated that the activation of the KOR antagonizes various MOR-mediated actions in the brain, including analgesia, tolerance, reward and memory processes (Pan, 1998). For instance, MOR facilitate dopamine release while dynorphin decreases dopamine levels (Donzanti et al., 1992). Moreover, it was shown that KOR can attenuate the acute behavioral effects of cocaine in rodents and can prevent the development of behavioral sensitization to cocaine as well as the decrease in self administration of cocaine (Heidbreder et al., 1993; Negus et al., 1997). Due to this opposing action, KOR may be the target for the treatment of addiction (Shippenberg et al., 1996).

1.2.3 Delta Opioid Receptors (DOR)

These receptors are involved in analgesia at the spinal and brain levels (Minami and Satoh, 1995). However, the details of its pharmacological characterization are still under examination. DOR can be found in the NAc, the limbic system and the dorsal horn of the spinal cord. DOR may play a role in meiosis, analgesia at the dorsal horn and hypotension (Hirsch, 1996). The activation of DOR can inhibit adenylyl cyclase and modulate membrane conductance of Ca^{2+} and K^{+} (North et al., 1987; Childers, 1991). The increase in K^{+} conductance and the decrease in Ca^{2+} conductance both

serve to reduce membrane excitability, control release of neurotransmitters, and thus may account for the analgesic properties of heroin (North et al., 1987; Chen et al., 1993).

Full-length transcripts of the DOR gene were discovered in the mouse brain and in no other tissues examined. Within the brain, the expression of the DOR gene is low. However, transcripts were found in particularly large amounts in the anterior pituitary and pineal glands (Evans et al., 1992). The abundant distribution of DOR in these regions suggests that they play a role in neuroendocrine function. Furthermore, the cloned DOR is targeted almost exclusively to axons (Dado et al., 1993; Arvidsson et al. 1995) where it is most likely to function presynaptically (Glaum et al., 1994). Befort and colleagues mapped the hDOR gene to chromosome 1p34.3-36.1 by isotopic in situ hybridization and the homologous gene to mouse chromosome 4 (Befort et al., 1994). Delta-selective agonists have been shown to modulate dopamine release in the NAc, thus affecting locomotor activity and self-administration behaviour (Devine and Wise, 1994). The antisense oligodeoxynucleotide to DOR attenuates morphine dependence in mice, suggesting that DOR may be involved in the development of morphine dependence and tolerance (Suzuki et al., 1994; Fundytus et al., 1995). An association between a T921C polymorphism of the DOR gene and heroin dependence in a German population has been reported (Mayer et al., 1997). It was suggested that the C allele could increase the risk for heroin dependence. However, the functional significance of this SNP has not been established.

1.3 Dopamine Receptors

Dopamine receptors can be classified into two main types, D1-like and D2-like. D1-like dopamine receptors consist of D1 and D5 dopamine receptors, which activate the enzyme adenylyl cyclase and increase intracellular levels of cAMP. D2-like dopamine receptors include D2, D3 and D4 dopamine receptors that inhibit adenylyl cyclase and decrease intracellular levels of cAMP (Kebabian and Calne, 1979). D2-like dopamine receptors also activate K^+ channels and exert an inhibitiory influence on Ca^{2+} channel activities (Vallar and Meldolesi, 1989). Both D1- and D2-like dopamine receptors belong to a large superfamily of neurotransmitter and hormone receptors that are coupled to their specific effector functions via G-proteins (Dohlman et al., 1991).

The D2 dopamine receptor (DRD2) has been implicated in many addiction behaviours such as alcoholism (Noble et al., 1998), nicotine dependence (Comings et al., 1996) and cocaine dependence (Comings et al., 1994). DRD2 can be found in the caudate putamen, NAc and olfactory tubercle. In addition, DRD2 mRNA is found in the dopaminergic cell bodies within the substantia nigra pars compacta and VTA (Sibley and Monsma, 1992).

The human DRD2 gene was cloned by Grandy et al in 1989. The DRD2 gene is localized to chromosome 11q22-23. The human DRD2 contains an additional 29 amino acids in its putative third cytoplasmic loop as compared with the rat gene. The coding sequence is interrupted by 6 introns (Grandy et al., 1989). The DRD2 gene extends over 270 kb and an intron of approximately 250 kb separates the putative first

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exon from exons encoding the receptor protein (Eubanks et al., 1992).

The contribution of the A1 allele of DRD2 gene to alcoholism has been investigated extensively in recent years. In 1990, Blum's group demonstrated that there is an association between A1 allele of the DRD2 gene and alcoholism. In a blinded study, Blum et al. showed that the presence of A1 allele of the DRD2 gene correctly classified 77% of alcoholics, and its absence classified 72% of non-alcoholics. The polymorphic pattern of this receptor gene suggests that a gene that confers susceptibility to at least one form of alcoholism is located on the q22-q23 region of chromosome 11 (Blum et al., 1990). Although a number of studies did not replicate the association between DRD2 gene and alcoholism, at least seven independent meta-analyses on a large number of Caucasian alcoholics and controls have confirmed a strong association of the A1 allele of DRD2 gene with alcoholism (Berman and Noble, 1997). Apart from *Taq*I A polymorphism, a *Taq*I B polymorphism being shown to be associated with alcoholism (Schuckit, 1986), and has also been shown to be associated with cocaine dependence (Noble et al., 1993) and polysubstance abuse (Uhl et al., 1993). As a result, it was suggested that subjects with A1 allele may compensate for the deficiency in their dopaminergic system by drinking alcohol and abusing drugs that are known to increase the brain's dopamine levels.

Twins studies have reported that there is a higher concordance rate for alcoholism among monozygotic twins than in dizygotic twins (Pickens et al., 1991). Other evidence indicated that adopted children with alcoholic birth parents were four times more likely to become alcoholic than those adopted children with non-alcoholic birth parents (Schuckit, 1986). These studies further showed that there is a strong genetic

factor that is associated with alcoholism and perhaps other substances of abuse.

In animal studies, it was shown that the D2 receptor-deficient mice were akinetic and bradykinetic in behavioral tests and showed significantly reduced spontaneous movements (Baik et al., 1995). Maldonado and colleagues found that there was a suppression of rewarding behavior with morphine in DRD2 knock-out mice. But these knockout mice displayed normal response when food was used as a reward (Maldonado et al., 1997).

Further to this, the long form of the D4 dopamine receptor (DRD4) exon III repeat polymorphism has been linked to novelty seeking personality traits (Ono et al., 1997) and opioid dependence (Kotler et al., 1997; Haim et al., 1998). The 7-repeat allele is significantly associated with the opioid dependence. Moreover, the personality trait of novelty seeking is also more pronounced in opioid abusers (Kotler et al., 1997). Based on these studies, further investigation into the association of dopamine receptor genes and opioid dependence is warranted.

1.4 Dopamine Transporter (DAT)

DAT acts to transport the released dopamine back into the presynaptic terminals for the next cycle. DAT is preferentially localized to the plasma membrane of medium-small diameter dendrites that contain the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH). These observations suggest that DAT may play a role in the removal of extracellular dopamine and psychostimulants that are bound to the

dendrites within the VTA and cortical and striatal axon terminals (Nirenberg et al., 1997b). The expression of DAT is slightly lower in the prefrontal cortex than in the striatum (Sesack et al., 1998). However, it remains unclear whether the dopaminergic neurons in the VTA showed a difference in DAT expression.

The involvement of DAT has been implicated in Parkinson disease, Tourette syndrome and cocaine and nicotine dependence. In 1992, the human DAT cDNA was cloned by Vandenbergh et al. and displayed a novel 40-base repetitive element with variable numbers of the repeat, ranging from 3 to 11 copies, in the 3-prime untranslated region (Vandenbergh et al., 1992). Animal studies demonstrated that the development of alcoholism is associated with the dopaminergic system in the striatum and the limbic system (Tiihonen et al., 1995). These authors showed that the striatal DAT density was markedly lower in non-violent alcoholics than in healthy controls, while slightly higher DAT densities were reported among violent alcoholics than controls. Thus svariants of the DAT gene may influence the vulnerability to alcoholism and other psychiatric diseases.

In 1999, Lerman et al demonstrated that there was association of allele 9 of the DAT gene polymorphism between lack of smoking, late initiation of smoking and length of quitting attempts (Lerman et al., 1999). Furthermore, this polymorphism was also associated with low scores for novelty seeking which was suggested to be the most significant personality correlated with smoking cessation. It was also hypothesized that individuals that carried this polymorphism may have altered dopamine transmission, which reduces their novelty and reward-seeking behaviours (Sabol et

al., 1999).

1.5 Gamma-Aminobutyric Acid (GABA) Receptors

GABA A (GABAA) receptors consist of 6 α forms (α1, α2, α3, α4, α5, α6), 4 β forms (β1, β2L, β2S, β3), 5 γ forms (γ1, γ2L, γ2S, γ3, γ4), 1δ form, 1π form, 1ε form and 3ρ forms (ρ1, ρ2, ρ3,) (Barnard et al., 1998). Heteropentamers with each subunit contribute to the wall of the central ion channel. Each subunit consists of a putative large N-terminal extracellular domain and 4 transmembrane domains with a large intracellular loop between the third and fourth membrane-spanning regions. The subunit combination of α1β2γ2 constitutes the major GABAA receptor subtype in the brain (Olsen and Tobin, 1990). The GABAA receptor subunits share a low amino acid sequence homology with the subunits of the nicotinic acetylcholine receptors, although both of them are in the same superfamily of the transmitter-gated ion channels (Barnard, 1992).

Benzodiazepines exerts their actions in the CNS through binding to the GABAA receptor complex. It was reproted that a γ subunit was needed for a GABAA receptor isoform to interact with benzodiazepines to enhance GABA responses (Pritchett et al., 1989). Moreover, the nature of the α subunit was also thought to determine the benzodiazepine site specificity (Wieland and Luddens, 1994).

In 1992, the GABA receptor γ2 subunit (GABRG2) gene was mapped to the immediate vicinity of the GABA receptor α1 subunit (GABRA1) gene on the long

arm of chromosome 5 (Warrington et al., 1992). These two genes were 6 cR apart on the radiation hybrid map. Wilcox et al. localized the γ2 gene to 5q31.1-q33.2 (Wilcox et al., 1992). In the γ2 knockout mice study, it was found that the γ2 subunit serves no essential function for the assembly, transport and insertion into the cell membrane of the GABAA receptors. However, it is essential for the establishment of normal signal transduction characteristics and in single channel conductance (Gunther et al., 1995). It has been reported that two novel polymorphisms in GABAA receptor β2 and α6 subunit genes on human chromosome 5q33 were associated with alcohol dependence in a Scottish population. Although the *NciI* polymorphism at the GABRG2 gene was also examined, no significant association was found between this polymorphism and alcohol dependence in this Scottish population (Loh et al., 1999). Furthermore, a significant association was reported between GABAA receptor β3 subunit gene (GABRB3) and alcoholism in a Caucasian population. In addition, when the DRD2 and the GABRB3 variants are combined, the risk for alcoholism is more robust than when these variants are considered separately (Noble et al., 1998).

1.6 Mesocorticolimbic Pathway

Two major types of research paradigms have aided in the elucidation of the brain structures and neuronal tracts associated with reward. First, intracranial self-stimulation (ICSS) experiments are widely used to study brain regions that appear to mediate reward. Secondly, physical or chemical lesions of specific brain areas have been utilized to study the influence of specific brain areas on drug self-administration behaviours (Niesink, 1999). These studies have indicated that specific limbic

structures in the brain appear to be associated with the reward pathway of the brain (Wise, 1990; Koob, 1992). The medial forebrain bundle, NAc, VTA, the lateral and ventromedial nuclei of the hypothalamus and the medial prefrontal cortex serve as core structures of the mesocorticolimbic pathway. Several other areas of the brain also provide input to the mesocorticolimbic pathway concerning emotional and motivational variables. These predominately limbic regions include the septum, amygdala and thalamus. Furthermore, other areas of the brain, which do not mediate reward, are involved in the translation of that experience into motor activity. For instance, the basal ganglia and cerebellum are responsible for the control of fine voluntary and learned motor control when the reward pathway is stimulated (Niesink, 1999).

The cell bodies of the mesocorticolimbic system originate in the VTA and project to the NAc, septal region, amygdala, frontal cortex and olfactory tubercle (Van Ree et al., 1999). It was speculated that the hypothalamus is also mediated indirectly by components of the mesocorticolimbic pathway. Drugs of abuse are able either directly or indirectly to innervate the reward system to produce a reinforcing experience. For example, the reinforcing effects of heroin appear to be mediated in part by the VTA and NAc (Self et al., 1995).

The stimulation of dopamine transmission in the mesocorticolimbic system is a fundamental property of drug addiction. Lesions of the dopaminergic system of the mesocorticolimbic pathway reduce drug self-administration, most often without influencing other distinct drug effects such as effects on locomotion and the

development of tolerance or physical dependence (Spanagel and Weiss, 1999). Brain microdialysis studies reveal that drugs of abuse preferentially increase dopamine release in the limbic areas, especially the NAc (Johnson and North, 1992; Pontieri et al., 1995). Tthe reinforcing effects of opiates have been associated with dopaminergic neurons in the mesocorticolimbic pathway. Lesions of the VTA or the NAc reduce opiate reinforcement without influencing the development of tolerance. Microinjections of opiates into the VTA also produce conditioned place preference, which suggests that VTA may contribute to the reinforcement of opiates (Van Ree et al., 1999).

Serotonergic neurotransmission in the medial forebrain bundle has also been found to influence the reward mechanism. Serotonin suppresses ICSS behaviour and serotonergic antagonists facilitate this behaviour (Niesink, 1999). The GABAergic system also potentiates reward. First the GABAergic and dopaminergic neurons exhibit significant interactions in the limbic system. GABA interneurons in the VTA synapse with dopaminergic neurons, these GABAergic interneurons normally serve to inhibit the firing of dopaminergic neurons. However, when morphine binds to the mu opioid receptors located on the GABAergic interneurons and GABA release is inhibited, this produces a disinhibition of dopaminergic neurotransmission which in turn causes the increased release of dopamine (Spanagel et al., 1990; Johnson and North, 1992).

1.6.1 Neural Substrates of Drug Reinforcement

There is also a role for the mesocorticolimbic dopamine system in the priming effects of opiate and psychostimulant drugs on relapse of drug-seeking behavior. This system is therefore also a major neural substrate of drug-reinforcement (Wise and Bozarth, 1987; Di Chiara and Imperato, 1988; Koob and Bloom, 1988). Morphine injections increase the firing of ventral tegmental dopamine neurons and thus increase the release of dopamine from nucleus accumbens terminals of the mesocorticolimbic dopamine system (Leone et al., 1991; Grant and Sonti, 1994). Moreover, these theories are consistent with several early studies which found that microinfusion of amphetamine directly into the NAc caused local dopamine release and reinstated heroin-seeking behaviour (Stewart and Vezina, 1988). Similarly, application of morphine directly into the VTA increases the release of dopamine in the NAc (Leone et al., 1991; Johnson & North, 1992). However, injections of morphine into other brain regions rich in opiate receptors are ineffective. Further evidence has shown that dopamine is involved in relapse. Several studies have found that directly acting dopaminergic agonists are powerful inducers of both cocaine- and heroin-seeking behaviour (Wise et al., 1990; Self et al., 1996). Conversely, the priming effects of heroin (Shaham and Stewart, 1996), amphetamine (Ettenberg, 1990) and cocaine (Self et al., 1996), are blocked by the administration of dopamine antagonists. It is clear from these studies that drug-induced dopamine release in the NAc is both necessary and sufficient for opiates and psychostimulants to induce a relapse of drug-seeking behaviour.

1.6.2 Molecular and Cellular Basis of Addiction

Addiction is a complex phenomenon with important psychosocial causes and consequences. However, it also involves a biological process. After a long period of drug exposure, the functioning of neurons are altered which in turn alters the functioning of the neural circuits where those neurons operate. Eventually, this leads to complex behaviours such as dependence, tolerance, sensitization and craving that are characteristics of the addicted state (Kreek, 1996; Wise, 1996; Koob and Le Moal, 1997).

The mechanism of the molecular adaptation of neurons to chronic opiate exposure has been well established. There is an up-regulation of the cAMP pathway in response to repeated opiate administration (Nestler, 1992; Nestler et al., 1993). This up-regulation involves a rise in the concentrations of adenylyl cyclase, cAMP-dependent protein kinase A (PKA), and other components of this signaling pathway, including the DA synthesizing enzyme TH. Up-regulation of the cAMP pathway would oppose acute opiate inhibition of the pathway and also would represent a form of physiological tolerance. Upon removal of opiates, the up-regulated cAMP pathway would become fully functional and contribute to features of dependence as well as withdrawal (Sharma et al., 1975; Collier, 1980; Nestler, 1992).

There is direct evidence to support this model in the neurons of the locus coeruleus, the major noradrenergic nucleus in the brain. These neurons regulate attentional states and the activity of the autonomic nervous system and have been implicated in somatic

opiate withdrawal (Koob et al., 1992; Maldonado, 1997).

Acute opiate administration inhibits locus coeruleus neurons by increasing the conductance of an inwardly rectifying potassium channel through coupling with the $G_{i/o}$ G-protein, as well as by reducing a sodium-dependent inward current through coupling with $G_{i/o}$ protein. Consequently, the inhibition of adenylyl cyclase results. Reduced concentrations of cAMP decrease PKA activity and the phosphorylation of the responsible channel or pump is also affected. Inhibition of the cAMP pathway also decreases phosphorylation of numerous other proteins and in turn modifies many additional processes in the neuron. For instance, it reduces the phosphorylation state of cyclic AMP response-element binding protein (CREB), one of the major cAMP-regulated transcription factors in the brain, which may initiate some of the longer-term changes in locus coeruleus function (Nestler and Aghajanian, 1997).

In contrast to the acute administration of opiates, chronic administration of opiates in the locus coeruleus is caused by the up-regulation of the cAMP pathway (Nestler and Aghajanian, 1997). Chronic morphine increases concentrations of types I and VIII adenylyl cyclase (Matsuoka et al., 1994; Lane-Ladd et al., 1997), PKA catalytic and regulatory type II subunits and several phosphoproteins including CREB. As a result, the intrinsic excitability of locus coeruleus neurons is increased by enhanced activity of the cAMP cascade and the sodium-dependent inward current, which contributes to tolerance, dependence and withdrawal. The up-regulation of type VIII adenylyl cyclase is mediated by CREB (Nestler and Aghajanian, 1997). A reduction in CREB concentration, which is achieved by infusion of antisense oligonucleotides to CREB,

blocks the morphine-induced increase in this enzyme. In contrast, up-regulations of type I adenylyl cyclase and PKA subunits are not affected by this treatment. Accordingly, antisense oligonucleotide treatment partially attenuates the activation of locus coeruleus neurons seen during withdrawal, as well as the severity of certain opiate withdrawal behaviours (Lane-Ladd et al., 1997). This is consistent with the observation that mutant mice deficient in CREB showed attenuated opiate withdrawal. Experiments with transgenic mice have implicated the transcription factor CREB as being critical to the changes produced by chronic opioid treatment (Maldonado et al., 1996). Symptoms of opioid withdrawal are markedly reduced in mice missing two CREB proteins.

In response to opiates, cells in the VTA and NAc also undergo plastic changes. Upregulation of the cAMP pathway occurs in neurons of the NAc in response to chronic administration of opiates, cocaine or alcohol (Terwilliger et al., 1991; Schoffelmeer et al., 1996). However, it remains unclear which of several cell types within this region mediate this adaptation. There is preliminary evidence which implicates the cAMP pathway in the VTA (Bonci and Williams, 1996; Tolliver et al., 1996) and the periaqueductal gray, a brainstem region that contains major serotonergic nuclei, in opiate withdrawal (Jolas and Aghajanian, 1997). Biochemical and electrophysiological evidence suggests that chronic opiate exposure leads to an up-regulated cAMP pathway in these brain regions, specifically within the GABAergic neurons that innervate the dopaminergic and serotonergic cells (Bonci and Williams, 1996; Tolliver et al., 1996; Jolas and Aghajanian, 1997). The up-regulation of the cAMP pathway would lead to increased GABA release during

withdrawal and thereby to a reduction in the firing of dopaminergic and serotonergic neurons. The former could account for the reduction in dopaminergic neurotransmission from the VTA to the NAc that occurs during early phases of drug withdrawal. In addition, it is thought to contribute to associated aversive states such as stress and anxiety (Kuhar and Pilotte, 1996; Koob and Le Moal, 1997).

Following continued drug reinforcement, a sustained increase in drug-seeking behaviour is exhibited by the individual for a prolong period when drug exposure has ceased. This persistent drug-craving behaviour may be caused by long-lasting adaptations in the brain's reward system. Chronic exposure to morphine, cocaine, or even alcohol, produces common intracellular biochemical adaptations in the VTA and NAc (Nestler, 1992; Nestler et al., 1993; Self et al., 1996). These adaptations consist of an increase in the level of TH in the VTA, and a decrease in the phosphorylation state of TH in the NAc (Beitner-Johnson and Nestler, 1991; Ortiz et al., 1995). These adaptations are accompanied by decreases in the level of neurofilament (NF) proteins in the VTA (Beitner-Johnson et al., 1992). Furthermore, there is an up-regulation of glial fibrillary acidic protein (GFAP) in the VTA in the case of chronic morphine and ethanol exposure (Beitner-Johnson et al., 1993; Ortiz et al., 1995).

1.6.3 Intracellular Substrates of Relapse

Drug craving and relapse are triggered by dopaminergic activation of the D2 receptors on NAc neurons. D2 receptors inhibit adenylyl cyclase activity by coupling inhibitory G-proteins, leading to decreased levels of cAMP. Reduced cAMP levels, in turn,

reduce the activity of PKA which otherwise would phosphorylate specific substrate proteins such as the voltage-gated neuronal sodium channels which are inactivated following repeated exposure to cocaine (White and Zhang, 1996). It is possible that these sodium channels are reactivated following acute inhibition of PKA activity (Li et al., 1992; Schiffmann et al., 1995; Smith and Goldin, 1996) and this event contributes to relapse of cocaine-seeking behaviour. Eventually, D2 dopamine receptor activation could increase the excitability of D2-containing neurons within the NAc, an effect hypothesized to contribute to drug craving and relapse. On the contrary, D1 dopamine receptors could activate the cAMP pathway via G-proteins that stimulate adenylyl cyclase. Thus, D1 dopamine receptors modulate neuronal sodium channels via PKA-dependent phosphorylation (Li et al., 1992; Schiffmann et al., 1995). PKA-mediated phosphorylation of neuronal sodium channels raises their threshold for activation, thereby reducing membrane excitability. This is one possible mechanism to explain the attenuation of cocaine priming produced by D1 agonists.

1.7 Environmental Factors in Drug Addiction

The availability of heroin on the street plays a vital role in the development of an individual's addiction. However, the availability of heroin alone is not enough to get a person induced to try the substance. Consider the historical cases of India and Turkey, where high quality opium was grown in abundance for years. Those societies never encountered the addiction problems of the nations they supplied. In the United States, although opiates were widely available to all population groups, some population groups have had much higher addiction rates than others. Therefore, instead of

availability, it seems that personality, peer group pressure and social learning may contribute to making the individual more susceptible to heroin addiction (Zackon, 1992; Hofler et al., 1999). Heroin users frequently demonstrate the kind of thinking and behaviour that psychiatrists call sociopathic. A sociopath is considered to lack the instincts that are essential for forming caring human relationships and is resistant to the lessons of experience (Brochu, 1997). Sociopaths are manipulative and devious, and always likely to blame others for the problems caused by their own behaviour (Zackon, 1992). One psychological condition widely seen in heroin addicts is low self-esteem (Hofler et al., 1999; Taylor, 2000). They believe that they are a failure or are incompetent. They have feelings of hopelessness, and sometimes have psychological depression or schizophrenia. Some psychologists have said that heroin addicts suffer from hypophoria, a kind of depression that is specifically related to low self-esteem. Another common observation is that heroin addicts often seem to be strangely dependent on one family member or on the whole family for emotional support and acceptance, or develop an unusual attachment to a spouse or lover. Some theorists interpret this behaviour as signaling problems with individuation. This is a maturation process of establishing one's own sense of identity and one's capacity for independent choice and action. Since many addicts begin using drugs in their adolescence, one possibility is that a very troubled individuation process made them prone to use drugs like heroin to get immediate feelings of warmth and security. Many addicts confirm that when they began to use heroin they found that it gave them the feelings of security and confidence they lacked; that the drug made them feel able to handle inner conflicts, personal relationships and sexual intimacy (Zackon, 1992). Furthermore, stress and in particular emotional distress might increase the

development of opiate-self administration (Shaham et al., 1992; Shaham and Stewart, 1994).

Next to availability, the key social factor that determines whether a person will use drugs is the influence of peers. Drug availability often interacts with peer group pressure (Wong et al., 1997; Farrell and White, 1998). Users usually get their first drugs from their friends. Of course, not everyone follows the example of others, and often a young person will have several distinct peer groups with which to associate. Moreover, the apparent power of peer influence suggests to many social psychologists that addiction may have a social learning component. It is said that through social learning, less experienced peers are not just offered heroin or encouraged to use it, but are actually taught to use it and appreciate it. And through the example and approval of more experienced peer users, they learn the lifestyle of addiction. Therefore, social theorists as well as personality theorists view peer influence as a contributor to a propensity for using drugs (Zackon, 1992).

1.8 Genetic Factors in Drug Addiction

The etiology of drug abuse is a complex interplay of psychosocial and biological factors. Genetic factors also contributed to an individual's vulnerability to drug abuse. At present, there are no genes shown to be unique in causing drug abuse. Instead, there are genes that alter the normal function of the CNS as manifested by a wide range of interrelated impulsive, compulsive, addictive, affective and anxiety behaviours (Comings, 1994; Comings et al., 1996). One of the outcomes or associated

behaviours, is the attempt to cope with these disorders through substance abuse. This means that the genetics of any one of these interrelated behaviours has relevance to drug abuse (Comings, 1996).

One of the major players in vulnerability to drug abuse in human subjects is the brain reward system that is composed of dopaminergic neurons. Recently, the DRD2 receptors were investigated in order to examine whether the variations in the prevalence of different forms of the DRD2 gene in drug addicts may participate in the susceptibility to drug addiction. The frequency of the *Taq*I A1 allele of DRD2 ine alcoholics (Noble, 1994; Blum et al., 1995; Noble et al., 1998), cocaine abusers (O'Hara et al., 1993; Comings et al., 1994) and nicotine abusers (Noble et al., 1994; Comings, 1996) has been compared with that of controls. It was found that the frequencies of the A1 allele in the alcoholics, cocaine abusers and nicotine abusers were significantly higher than in controls. Noble and coworkers reported that in 57 severe alcoholics, when compared to 45 controls, a significant increase was found in the prevalence ($p=1.7x10^{-5}$) and frequency ($p=1.6x10^{-5}$) of the DRD2 A1 allele. Moreover, a significant progressive increase was observed in A1 allele prevalence ($p=3.1x10^{-6}$) and frequency ($p=2.7x10^{-6}$) in the order of 45 controls, 114 less severe alcoholics and 57 severe alcoholics. In the same study, it was reported that the GABAA receptor β3 subunit gene was also involved in alcoholism. In 57 severe alcoholics, when compared with the 45 controls, a significant decrease was found in the prevalence ($p=4.5x10^{-3}$) and frequency ($p=2.7x10^{-2}$) of the major G1 allele of the GABAB3 receptor. Moreover, a significant progressive decrease was noted in the G1 allelic prevalence ($p=2.4x10^{-3}$) and frequency ($p=1.9x10^{-2}$) in controls, less severe

alcoholics and severe alcoholics (Noble et al., 1998). Furthermore, it has been reported that two novel polymorphisms in GABAA receptor β2 and α6 subunit genes were associated with alcohol dependence in a Scottish population (Loh et al., 1999).

Family, twin and adoption studies give the greatest source of information for examining the role of genetic factors in drug addiction, especially alcoholism. Merikangas and colleagues showed that there was an 8-fold increased risk of drug disorders among the first degree relatives of probands with drug disorders across a wide range of specific substances like opioids, cocaine, cannabis and alcohol. There was also evidence of specificity of familial aggregation of the predominant drug of abuse. Rates of abuse or dependence on hard drugs were greatest among the relatives of probands with opioid disorders (14.5%), moderately elevated among relatives with cocaine or cannabis disorders (9.6% and 8.4% respectively) and lowest among relatives of probands with alcoholism (4.4%) when compared with relatives of controls (1.2%) (Merikangas et al., 1998). These findings supported the postulate that there may be some genes that predispose to drug abuse. Pickens and colleagues in a twin study of alcoholism showed a concordance rate among 114 male monozygotic twins of 63.4% versus 43.8% for dizygotic twins (p=0.05) (Pickens et al., 1991). Tsuang and colleagues studied 3372 male twin pairs from the Vietnam Era Twin Registry and showed that heroin abuse had the largest amount of unique genetic variance (38%) and the least amount of shared genetic variance (16%) when compared with other drugs like marijuana and amphetamines. It was concluded that heroin had a larger genetic influence unique to itself than any other drugs of abuse (Tsuang et al., 1998).

From adoption studies, Goodwin and coworkers noted that the sons of alcoholics were about 4 times more likely to be alcoholics than sons of non-alcoholics and that being raised by either non-alcoholic adoptive parents or by biological parents did not affect this increased risk (Goodwin, 1979). In view of this, it was suggested that genetic factors influence the risk for alcohol and drug dependence.

Recent evidence that demonstrated that genetic factors may have a role to play in heroin addiction came from association studies between the A118G polymorphism of the hMOR gene and opioid dependence (Bond et al., 1998). Another polymorphism of the C17T variant of hMOR was also found to be more prevalent among 55 opioid-dependent subjects and 51 controls, although only at a marginal significance level ($p=0.054$) (Berrettini et al., 1997). The hDOR gene T921C polymorphism was also shown to be associated with heroin dependence in a German population (Mayer et al., 1997). All this evidence suggests that individuals with various genetic compositions have different extents of vulnerability to substance abuse.

1.9 Aim of Project

The genetic contribution in addictive behaviours has been extensively investigated in recent years. Polymorphisms of receptor genes such as DRD2 (Lawford et al., 1997; Noble, 1998), DRD4 (Kotler et al., 1997; Tomitaka et al., 1999), GABA (Loh et al., 1999), DAT (Lerman et al., 1999), hMOR (Berrettini et al., 1997; Bond et al., 1998) and hDOR (Mayer et al., 1997) have been shown to be associated with alcoholism,

cocaine dependence, nicotine dependence, heroin dependence or gambling in different populations.

The aim of the present study was to explore if there is any association between a selected number of potential candidate gene polymorphisms and heroin dependence in the Hong Kong Chinese population. The advantage of an association study is that no assumption is made for the mode of inheritance, penetrance and age of onset of addictive behaviour. In the present study, a total of 200 heroin-dependent subjects were examined. The subjects were derived from two sources: heroin-dependent individuals who sought treatment at the Substance Abuse Clinic at the Prince of Wales Hospital, and heroin-dependent volunteers identified by snowball recruitment. All subjects were examined and assessed by a psychiatrist who specialized in substance abuse and all heroin-dependent subjects conformed to the criteria for opiate dependency as defined by DSM IV. Heroin-dependent subjects with a history of alcohol or substance dependence other than heroin were excluded from the study. Urine specimens obtained from each subject were analyzed to confirm heroin use and to exclude polysubstance abuse. A total of 97 control subjects were also used in this study. They were healthy blood donors at the Prince of Wales Hospital. Those who had a history of drug or alcohol dependence, smoking, gambling, or psychiatric illnesses were excluded.

DNA was extracted from peripheral blood samples followed by genotyping using the restriction fragment length polymorphism and denaturing HPLC (dHPLC) methods. In this study, the A118G polymorphism at exon 1 of the hMOR gene, C1031G

polymorphism at intron 2 of the hMOR gene, T921C polymorphism at exon 3 of the hDOR gene, *Taq*I A polymorphism of the DRD2 gene, 3' VNTR polymorphism of the DAT gene as well as the GABRG2 gene were examined. Since ethnic variance exists for the A118G hMOR exon 1 polymorphism (Bond et al., 1998), the present study attempted to establish whether such ethnic variance also exists in the Chinese population. Nucleotide sequence information of intron 2 (NCBI Entrez NID: g2655102) revealed a C1031G polymorphism of the hMOR gene. Similarly, the delta opioid receptor has been implicated in the modulation of dopamine release in the NAc (Devine and Wise, 1994) and the development of morphine dependence and tolerance (Suzuki et al., 1994; Fundytus et al., 1995) Nucleotide sequence information for exon 3 (NCBI Entrez NID: g497313) revealed a T921C polymorphism of the hDOR gene. Hence its role in heroin dependence was also investigated by Mayer et al. (1997). Since the *Taq*I A polymorphism of the DRD2 gene, 3' VNTR polymorphism of the DAT gene as well as the GABRG2 gene have been implicated in alcoholism and substance dependence, their role in heroin dependence in the Chinese population was also investigated. Any significant association between the polymorphisms of these candidate genes and heroin dependence was assessed using Yates chi-square test. The interaction between genotypes was also investigated by calculating the relative odds using logistic regression analysis. This analysis enabled the examination of a zero, a single or a double gene dosage of the variant gene that is carried by an individual, to assess the gene dosage of a variant gene affects the robustness of its vulnerability to heroin dependence.

In addition, all of the heroin-dependent subjects were interviewed, after training,

using the Addiction Severity Index (ASI) questionnaire that covers medical, employment, drug, legal, family history, family/social relationships and psychiatric status. It can be used to rate the severity of a wide range of problems in the subject's life and suggest how these problems must be addressed if treatment is to be successful. In order to reveal if there is any correlation among the factors of ASI, Pearson's regression analysis followed by the chi-square test was used. In addition, the heroin-dependent subjects were divided into a less or more severe group based on their indices 0-4 or 5-9 respectively. This was to investigate whether any correlations may exist between variant allelic frequencies and individual factors of ASI. Results from this may help to draw a link between the genetic and environmental factors that contribute to heroin dependence.

CHAPTER TWO MATERIALS AND METHODS

2.1 Recruitment of Subjects

2.1.1 Heroin-dependent Subjects

200 unrelated Chinese heroin-dependent abusers (185 males, 15 females) were recruited for this study. The patients were derived from two sources: the Substance Abuse Clinic at the Prince of Wales Hospital (PWH) (n=79) and snowball recruitment from The Society and Rehabilitation of Drug Abuse (n =121).

2.1.1.1 Phenotype Assessment

All patients were subjected to psychiatric assessment by the psychiatrist at the Substance Abuse Clinic at PWH using the Diagnostic and Statistical Manual IV (DSM IV) as the diagnostic instrument. DSM IV diagnostic criteria for substance abuse define a maladaptive pattern of substance use leading to clinically significant impairment or distress as manifested by three (or more) of the following, occurring at any time in the same 12-month period: i) Substance is often taken in larger amounts or over a longer period than intended; ii) Persistent desire or unsuccessful efforts to cut down or control substance use; iii) A great deal of time is spent in activities necessary to obtain the substance (e.g., visiting multiple doctors or travelling long distances), use the substance (e.g., chain smoking), or recover from its effects; iv) Important social, occupational, or recreational activities given up or reduced because

of substance abuse; v) Continued substance use despite knowledge of having a persistent or recurrent psychological or physical problem that is caused or exacerbated by the use of the substance.

With regular heroin use, tolerance develops. This means the abuser must use more heroin to achieve the same intensity or effect. As higher doses are used over time, physical dependence and addiction develop. With physical dependence, the body has adapted to the presence of the drug and withdrawal symptoms may occur if use is reduced or stopped. Withdrawal, which in regular abusers may occur as early as a few hours after the last administration, produces drug craving, restlessness, muscle and bone pain, insomnia, diarrhea and vomiting, cold flashes with goose bumps ("cold turkey"), kicking movements ("kicking the habit"), and other symptoms. Major withdrawal symptoms peak between 48 and 72 hours after the last dose and subside after about a week. Sudden withdrawal by heavily dependent users who are in poor health is occasionally fatal, although heroin withdrawal is considered much less dangerous than alcohol or barbiturate withdrawal.

Heroin-dependent patients were excluded if they had a history of alcohol or substance dependence other than heroin. Informed consent (written in Chinese) from all subjects was obtained prior to interview as well as blood and urine sample collection.

2.1.1.2 Establishment of Socio-demographic Data

All subjects were interviewed using the Addiction Severity Index (ASI). ASI is a structured clinical interview for the collection of reliable and valid data on areas

related to drug addiction (Grant 1992). It can be used to rate the severity of a wide range of problems in the subject's life. The problems identified in the assessment do not necessarily relate to the problem of substance abuse, but must be addressed for treatment to be successful. ASI covers areas including medical, employment, drug, legal, family history, family/social relationships and psychiatric status (see Appendix I). Subjects were asked to rate how severe each problem has been in the last 30 days and whether they considered it important to seek treatment for the problem at the time of interview. The scale starts at 0, meaning the subject is "not at all" bothered by the problem and ends at 4, meaning the subject is "extremely" troubled by the problem. The interviewer makes an independent assessment of the severity of each problem using a 10-point scale and calculates a severity rating. The scale starts at 0, meaning no problem exists and treatment is probably not necessary and ends at 9, meaning an extreme problem exists and treatment is a must. The subject's score is then used to adjust slightly the interviewer's assessment of the severity of the problem (see Appendix II). The family history section was not given a severity index, but family history of alcohol-, drug- and psychiatric-related illnesses were recorded. The technology transfer package developed by the National Institute on Drug Abuse contains all the materials necessary for interviewers to conduct their own training. The material contains two training videotapes which demonstrate proper use of the ASI; a training facilitator's guide suggesting practice exercises; and a resource and scoring manual which provides instructions for calculating severity scores, interviewing tips, and an item-by-item discussion of the instrument. The ASI has been used in large research studies including the Drug Abuse Treatment Outcomes Study (DATOS) and the National Treatment Improvement Effectiveness Study (NTIES).

ASI gives highly consistent results when used by different interviewers and composite scores have proven effective in measuring treatment outcomes accurately. The severity ratings have the advantage of providing a readily understandable 10-point scale for representing a client's problems and need for treatment in each area covered by the interview.

After the interview, a registered nurse and a medical officer collected a urine specimen and 6ml of blood sample respectively. The urine specimen was analyzed immediately using a urine kit (Triage ®Panel for Drug of Abuse, Biosite Diagnostics) to confirm heroin use and to exclude polysubstance use.

2.1.2 Control Subjects

A total of 97 control subjects (students, both academic and non-academic staff, 53 males, 44 females) were recruited from PWH as well as The Chinese University of Hong Kong. They were healthy blood donors with no history of drug or alcohol dependence, smoking, gambling or psychiatric illness.

2.2 DNA Extraction

Blood was drawn from the peripheral vein of the subject and anticoagulated with EDTA. The blood samples were stored at –70°C before extraction. DNA was extracted from peripheral blood samples with the Viogene Blood and Tissue Genomic DNA Miniprep System (Viogene Catalog no. GG1001, USA). First of all, 200μl

blood sample was pipetted into a 1.5ml microcentrifuge tube. 20µl of 20mg/µl proteinase K and 200µl buffer EX were added to the sample. The mixture was mixed immediately by vortexing for 20 seconds (sec). Afterwards, the mixture was incubated at 60°C for 20 minutes (min) to lyse the cells. The sample was further incubated at 70°C for 20 min to inactivate proteinase K. 210µl of isopropanol was then added to the sample and mixed by vortexing. After mixing, all the mixture was applied to the genomic DNA column (Viogene, USA) in a 2ml collection tube. The column, together with the 2ml collection tube, were centrifuged at 8000 rpm for 2 min. The filtrate was decanted and the column was placed back to the collection tube. 500µl wash buffer was added to the column and was centrifuged again at 8000 rpm for 2 min. Then the column was placed into a new collection tube. Another 500µl of wash buffer was applied prior to further centrifugation at 8000 rpm for 2 min and at 13000 rpm for a further 2 min to dry the column. The column was put into a new collection tube and DNA was eluted with 200µl of preheated ddH_2O by centrifugation at 8000 rpm for 2 min.

2.3 Genotyping

2.3.1 A118G Polymorphism in Exon 1 of the Human MOR (hMOR) Gene

The A118G polymorphism in exon 1 of the hMOR gene was analyzed using the restriction fragment length polymorphism (RFLP). The hMOR exon 1 RFLP was amplified using a forward primer: 5'-TCA GTA CCA TGG ACA GCA GC-3' and the reverse primer: 5'-GCA CAC GAT GGA GTA GAG GG-3'. 50 ng of purified DNA

was diluted into the PCR reaction mix consisting of PCR buffer (100mM Tris-HCl, pH 8.8 at 25°C; 500ml KCl; 0.8% Nonidet P40 and 15mM $MgCl_2$) 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 25 pmol of each forward and reverse primer and 1U AmpliTaq Gold™ polymerase (Perkin Elmer Applied Biosystems, USA) in a total volume of 50µl. PCR was carried out in the thermal cycler (Perkin Elmer Applied Biosystems, Model no. PCR 9700, USA). The amplification steps were: i) denaturation at 94°C for 13 min, ii) 35 cycles of denaturation at 94°C for 45 sec, iii) annealing at 62°C for 30 sec, iv) synthesis at 72°C for 30 sec and v) final elongation at 72°C for 7 min. After 35 cycles, the PCR products were detected in a 2% agarose gel. PCR products were then loaded into a 96-well plate and put into the WAVE™ DNA Fragment Analysis System (Transgenomic, Inc., USA) for genotyping. Denaturing HPLC (dHPLC) is a semi-automated method for detecting unknown DNA sequence variants. The sensitivity of the method is dependent on the temperature which is kept by the oven (Jones et al,. 1999). The stationary phase consisted of 2µm nonporous alkylated poly-styrene divinylbenzyl (PS-DVB) micropellicular matrix packed into a 50 x 4.6 mm column (Huber, 1998). A 10µl aliquot of each PCR product was then injected into the column and eluted at a flow rate of 0.9 ml/min with a mobile phase consisting of a mixture of buffer A and buffer B (Pirulli et al., 2000). Buffer A consisted of 0.1 M/l triethylammonium acetate (TEAA) solution, pH 7.0. Buffer B consisted of 0.1 M/l TEAA containing 25% acetonitrile. The eluted DNA fragments were detected at 260 nm. Heterozygous species that are generated have a 1:1 ratio of wild-type and mutant DNA. A mixture of hetero- and homoduplexes can be yielded when the PCR product is hybridized by heating to 95°C and cooled down slowly. The base mismatch on the heteroduplex

formed a linear region of the single-stranded DNA that has been used to separate the two species and predict the mutation. At increasing temperatures, the DNA starts to melt selectively in the region of the base mismatch of the heteroduplexes. As a result, heteroduplex molecules were eluted before the homoduplexes, two peaks were then subsequently formed (see Fig 2 in Section 3.3). Genotyping was carried out without knowledge of the affected status. Prior to each run, 10µl of 209 mutation standards (Transgenomic, USA) was injected to ensure the optimal condition of the WAVE™ DNA Fragment Analysis System for mutation screening.

2.3.2 C1031G Polymorphism in Intron 2 of the hMOR Gene

20 ng purified DNA was diluted into the PCR reaction mix consisting of PCR buffer (100mM Tris-HCl, pH 8.8 at 25° C; 500ml KCl; 0.8% Nonidet P40 and 15mM MgCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 25 pmol of each forward and reverse primer and 1U Taq polymerase (MBI Fermentas, NY, USA) in a total volume of 25µl. The C1031G polymorphism was detected by an artificial *Hinf*I RFLP analysis. Using a modified forward primer: 5'-GCT CTG GTC AAG GCT AA**G** AAT-3', in which nucleotide 1027 (underlined) was changed from an A to a G, thereby an artificial *Hinf*I restriction site was generated. This was combined with the reverse primer: 5'-GTA AGA GAG TAG GTT GGA CCA-3' to amplify a 145bp PCR fragment. PCR was carried out in a thermal cycler (Perkin Elmer). The amplification steps were: i) denaturation at 94°C for 5 min, ii) 35 cycles of denaturation at 94°C for 45 sec, iii) annealing at 62°C for 30 sec, iv) synthesis at 72°C for 45 sec and v) final elongation at 72°C for 7 min. 8µl of the 145 bp PCR

product was digested with 5U *Hinf*I (MBI Fermentas) restriction enzyme overnight at 37° C. The restriction-digested products were resolved on a 5% agarose gel stained with ethidium bromide. The fragment lengths of the C allele were 124 and 21 bp, while the fragment length of the PCR product remained a 145 bp when the G allele was present. Heterozygous CG allele was indicated by the presence of both the 124 bp and 145 bp bands (see Fig. 10 in Section 3.4).

2.3.3 T921C Polymorphism in Exon 3 of the Human DOR (hDOR) Gene

20ng purified DNA was diluted into the PCR reaction mix consisting of PCR buffer (100mM Tris-HCl, pH 8.8 at 25° C; 500ml KCl; 0.8% Nonidet P40 and 15mM MgCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 25 pmol of each forward and reverse primer and 1U Taq polymerase (MBI Fermentas) in a total volume of 25µl. The T921C polymorphism was detected by an artificial *BstE*II RFLP analysis. Using a forward primer: 5'-TTC GTC ATC GTC TGG ACG CT-3' and a modified reverse primer: 5'-GGT TGA GGC TGC TAT TGG **G**GT A-3' in which nucleotide 1158 (underlined) was changed from a C to a G, thereby an artificial *BstE*II restriction site was generated. These two primers were combined to amplify a 106bp PCR fragment. PCR was carried out in a thermal cycler (Perkin Elmer). The amplification steps were: i) denaturation at 94°C for 5 min, ii) 35 cycles of denaturation at 94°C for 45 sec, iii) annealing at 62°C for 45 sec, iv) synthesis at 72°C for 45 sec and v) final elongation at 72°C for 7 min. 8µl of the 106 bp PCR product was digested with 5U *BstE*II (New England BioLabs Inc.) restriction enzyme

overnight at 60°C. The restriction digest products were resolved on a 5% agarose gel stained with ethidium bromide. The fragment length of the T allele was 89 bp, while the fragment length of the PCR product remained at 106 bp when the C allele was present. Heterozygotes TC were indicated by the presence of both the 89 and 106 bp bands (see Fig. 12 in Section 3.5).

2.3.4 3'VNTR Polymorphism of DAT Gene

Genotyping was performed on 83 controls and 161 heroin-dependent subjects. 50ng purified DNA was diluted into the PCR reaction mix consisting of PCR buffer (100mM Tris-HCl, pH 8.8 at 25°C; 500ml KCl; 0.8% Nonidet P40 and 15mM MgCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 25 pmol of each forward and reverse primer and 1U Taq polymerase (MBI Fermentas) in a total volume of 25µl. The 3' VNTR polymorphism of the DAT gene was detected by a RFLP analysis using a forward primer: 5'- TGT GGT GTA GGG AAC GGC CTG AG-3' and a reverse primer: 5' - CTT CCT GGA GGT CAC GGC TCA AGG-3'. PCR was carried out in a thermal cycler (Perkin Elmer). The amplification steps were: i) denaturation at 94°C for 5 min, ii) 30 cycles of denaturation at 94°C for 30 sec, iii) annealing at 62°C for 30 sec, iv) synthesis at 72°C for 30 sec and v) final elongation at 72°C for 10 min. The size of PCR products ranged from 320bp to 520bp and these were resolved on a 4% agarose gel stained with ethidium bromide. The 6-repeat, 7-repeat, 9-repeat, 10-repeat and 11-repeat elements were represented by the band sizes 320bp, 360bp, 440bp, 480bp and 520bp respectively (see Fig.17).

2.3.5 *Taq*I A Polymorphism of the DRD2 Gene

Genotyping was performed on 89 controls and 174 heroin-dependent subjects. 50ng purified DNA was diluted into the PCR reaction mix consisting of PCR buffer (100mM Tris-HCl, pH 8.8 at 25°C; 500ml KCl; 0.8% Nonidet P40 and 15mM MgCl), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 25 pmol of each forward and reverse primer and 1U Taq polymerase (MBI Fermentas) in a total volume of 30µl. The *Taq*I A polymorphism was detected by RFLP analysis using a forward primer: 5'-GCT CTA TCT CCA ACT CTC ACA-3', and a reverse primer: 5'-AAG TCT ACT CAC CTC CAG GTA-3' to amplify a 310bp PCR fragment. PCR was carried out in a thermal cycler (Perkin Elmer). The amplification steps were: i) denaturation at 94°C for 5 min, ii) 30 cycles of denaturation at 95°C for 30 sec, iii) annealing at 56°C for 30 sec, iv) synthesis at 72°C for 1 min and v) final elongation at 72°C for 10 min. 10µl of the 310 bp PCR product was digested with 5U *Taq*I (MBI Fermentas) restriction enzyme for at least 8 hours (hr) at 65°C. The restriction-digested products were resolved on a 2% agarose gel stained with ethidium bromide. The fragment lengths of the A2 allele were 180 and 130bp, while the fragment length of the PCR product remained at 310 bp when the A1 allele was present. Heterozygous A1A2 allele was indicated by the presence of the 130 bp, 180bp and 310 bp bands.

2.3.6 *Nci*I Polymorphism of the GABRG2 Gene

Genotyping was performed on 96 controls and 170 heroin-dependent subjects..20ng purified DNA was diluted into the PCR reaction mix consisting of PCR buffer

(100mM Tris-HCl, pH 8.8 at 25°C; 500ml KCl; 0.8% Nonidet P40 and 15mM MgCl), 2.5 mM $MgCl_2$, 0.25 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 30 pmol of each forward and reverse primer and 1U Taq polymerase (MBI Fermentas, USA, NY) in a total volume of 30µl. The *Nci*I polymorphism of GABRG2 was detected by an artificial *Nci*I RFLP analysis. Using a modified forward primer: 5'-AGA AAT TTA CCA ACT GGT CTA GCC GG-3' in which nucleotide 3145 (underlined) was changed from an A to a C, thereby an artificial *Nci*I restriction site was generated. The reverse primer was: 5'- AAA TCA AAT ATT GTG TCA TGC TTA GT-3'. These two primers were combined to amplify a 287bp PCR fragment. PCR was carried out in a thermal cycler (Perkin Elmer). The amplification steps were: i) denaturation at 95°C for 4 min, ii) 40 cycles of denaturation at 95°C for 30 sec, iii) annealing at 50°C for 30 sec, iv) synthesis at 72°C for 40 sec and v) final elongation at 72°C for 10 min. 8 µl of the 287 bp PCR product was digested with 5U *Nci*I (New England BioLabs) restriction enzyme overnight at 60°C. The restriction digest products were resolved on a 2.5% agarose gel stained with ethidium bromide. The fragment lengths of the G allele were 263bp and 24bp,while the fragment length of the PCR product remained as 287bp when the A allele was present. Heterozygotes AG were indicated by the presence of the 24bp, 263bp and 287bp bands (see Fig. 18).

2.4 DNA Sequencing

DNA sequencing was employed to ensure the accuracy of the genotypes obtained. The PCR Clean Up-M system (Viogene, Catalog no. PF1001, USA) was used. Firstly, the PCR product was pipetted to a 1.5ml tube and 100µl of ddH_2O was added. Then

0.5ml of buffer PX was added to the sample. After mixing well, the mixture was transferred to the PCR Clean Up-M column in a 2ml collection tube and then centrifuged for 1 min. After centrifugation, the filtrate was discarded and the column was washed and centrifuged for 1 min twice with 0.5ml wash I buffer and 0.7ml wash II buffer respectively. The column was further centrifuged for an additional 3 min in order to remove the ethanol residue. Finally, the column was placed into a new 1.5ml tube and then 30μl of ddH_2O was added to the centre of the column. The tube was left to stand at room temperature for 1 min and DNA was eluted by centrifugation for 2 min. 5μl of purified PCR product was mixed with 1μl 3pmol/μl forward primer and 4μl of DNA Sequencing Kit dRhodamine Terminator Cycle Sequencing Ready Reaction (Perkin Elmer Biosystems, England). The sequencing reaction was carried out at 96°C for 10 sec, then 25 cycles for 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min and finally 4°C for 7 min. Prior to DNA sequencing, an AutoSeq ™ G-50 column (Amersham Pharmacia Biotech Inc, USA) was used to remove the excess dRhodamine Terminator from the completed DNA sequencing reaction. DNA sequencing was then performed in an automated ABI Prism 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, USA).

2.5 Statistical Analysis

2-tailed Yates chi square (χ^2) analysis was applied to compare the difference in allele and genotype frequencies between the heroin-dependent subjects and the controls. $P < 0.05$ was considered statistically significant. To examine the interaction between genotypes, Yates χ^2 test was also used to compare the frequencies of the combined

genotypes among control and heroin dependent subjects.

Logistic regression is a variation of ordinary regression, useful when the observed outcome is restricted to two values, which usually represent the occurrence or non-occurrence of some outcome event, (usually coded as 1 or 0, respectively). It produces a formula that predicts the probability of the occurrence as a function of the independent variables. In this study, logistics regression analysis was carried out by using SPSS to calculate the relative odds (RO) in determining the effects of the combined genotypes to see whether the difference in the dosage of variant genes that are carried by individuals will make the vulnerability to heroin dependence more robust or not (Noble et al., 1998). For instance, subjects who had the AA of A118G and CC of C1031G were scored as 0, this mean they had a zero dose of the susceptibility gene. Subjects who had the AA, AG of A118G or the CC, CG of C1031G were labeled as 1, this means they had a single dose of the susceptibility gene, while subjects who carried the AG, GG of A118G and CG, GG of C1031G were scored as 2, indicating that they had a double dose of the susceptibility gene.

To explore the correlation between the genotypes and the factors in ASI, a χ^2 test was also used. The heroin dependent subjects were divided into 2 groups: a less or a more severe group in 6 areas of ASI. Subjects who scored between 0-4 or between 5-9 were classified into the less or more severe group respectively. In addition, the correlation between factors in ASI was investigated by using $\chi 2$ test and Pearson correlation coefficient (r).

CHAPTER THREE RESULTS

3.1 Socio-demographic Data

3.1.1 Age of the Control and Heroin-dependent Subjects

The age of the control and heroin-dependent subjects ranged from 18 to 57 and 16 to 61 years respectively. The mean ages of the control and heroin-dependent subjects were 32.8±11.2 and 35.8±11.6 years respectively. When the age distribution was examined, it was found that 6 (6.2%), 42 (43.3%), 25 (25.8%), 15 (15.5%) and 9 (9.3%) of the control subjects aged 11-20, 21-30, 31-40, 41-50 and 51-60 years respectively (Fig. 1a). The age distribution of the heroin-dependent subjects was 28 (14%), 39 (19.5%), 54 (27%), 63 (31.5%), 14 (7%) and 2 (1%) of them aged 11-20, 21-30, 31-40, 41-50, 51-60 and 61-70 respectively (Fig. 1b).

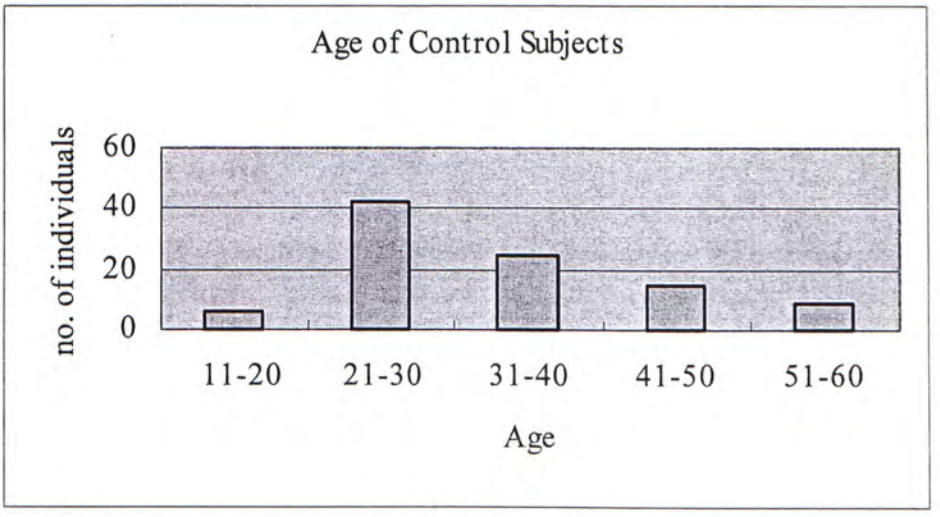
3.1.2 Educational Standard of the Heroin-dependent Subjects

The educational standards of the heroin-dependent subjects ranged from 0 to 19 years with a mean of 9.8±3.2 years. Amongst these subjects, 7 (3.5%) completed 0-3 years of education, 67 (33.5%), 119 (59.5%) and 7 (3.5%) subjects completed 4-9 (primary school), 10-13 (Form 1-3 of secondary school) and 14-19 (Form 4 to second year of University) years of education respectively (Fig. 2). Thus, 96.5% of subjects received a maximum education of up to Form 3 secondary school standard only.

Fig. 1a) Age distribution of control subjects (n=97).

1b) Age distribution of heroin-dependent subjects (n=200).

Fig.1a)


Age of Control Subjects
no. of individuals
60
40
20
0
11-20
21-30
31-40
41-50
51-60
Age

b)

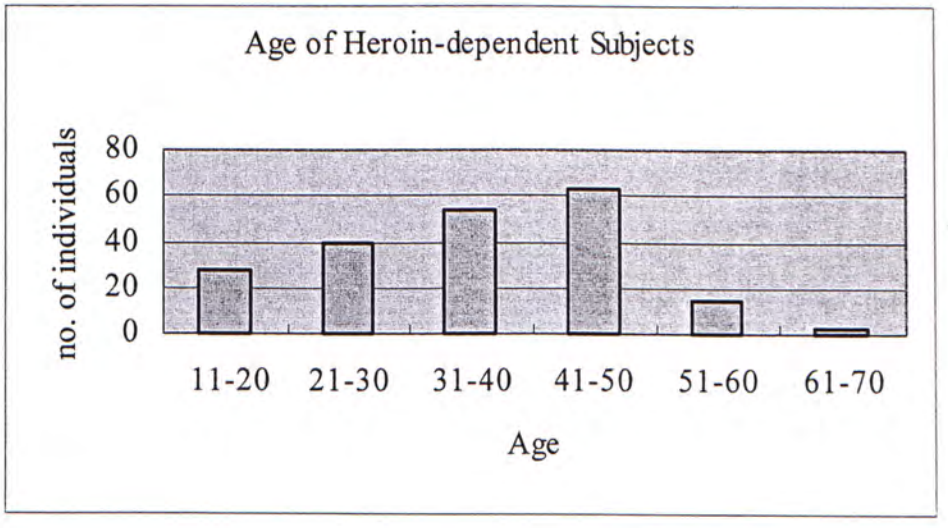
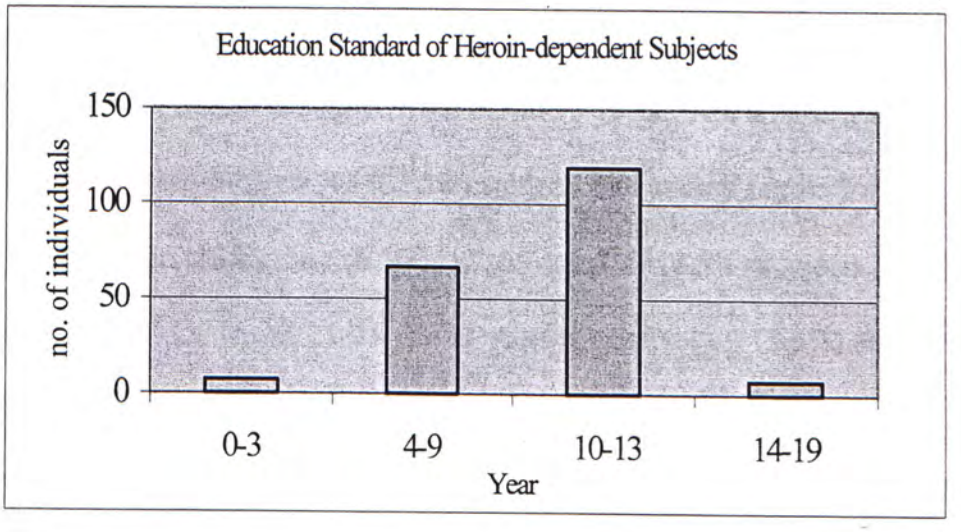

Age of Heroin-dependent Subjects
no. of individuals
80
60
40
20
0
11-20
21-30
31-40
41-50
51-60
61-70
Age

Fig. 2 The distribution of the educational standards in the heroin-dependent subjects (n=200).

Fig. 2


Education Standard of Heroin-dependent Subjects
150
100
50
0
no. of individuals
0-3
4-9
10-13
14-19
Year

3.1.3 Years of Heroin Use

The years of heroin use range from 0.5 to 35 years, with an average of 14±9.1years. Amongst these subjects, 47 (23.5%) subjects had abused heroin for less than 5 years, 38 (19%), 26 (13%), 53 (26.5%), 12 (6%) and 17 (8.5%) subjects had abused heroin for 6-10, 11-15, 16-20, 21-25, 26-30 years respectively. 7 (3.5%) subjects had abused heroin for over 30 years (Fig. 3).

3.2 Addiction Severity Index (ASI)

Using the ASI (see Section 2.1.1.2; Appendix 1), heroin-dependent subjects in this study were interviewed in areas such as medical, employment, drug, legal, family/social relationships and psychiatry status and background. In each area, an index was given to rate the severity.

3.2.1 ASI – Medical

Fig. 4a shows that among the 200 heroin-dependent subjects interviewed, 151 subjects (75.5%) scored 0 for the medical category of the ASI. 20 subjects (10%) scored 1, the remaining 29 (14.5%) subjects scored between 2 and 8. No individual scored a 9.

Fig. 3 The distribution of the no. of years of heroin use in the heroin-dependent subjects (n=200).

Fig.3

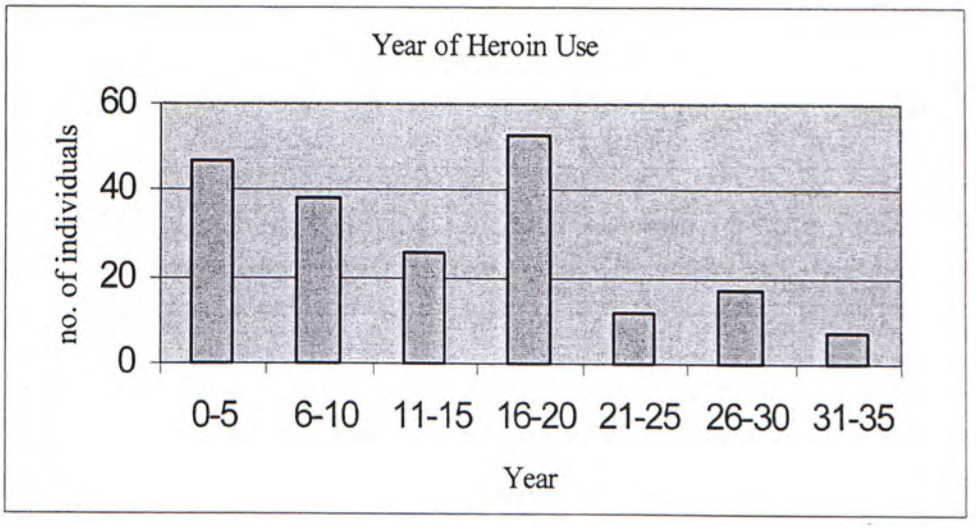
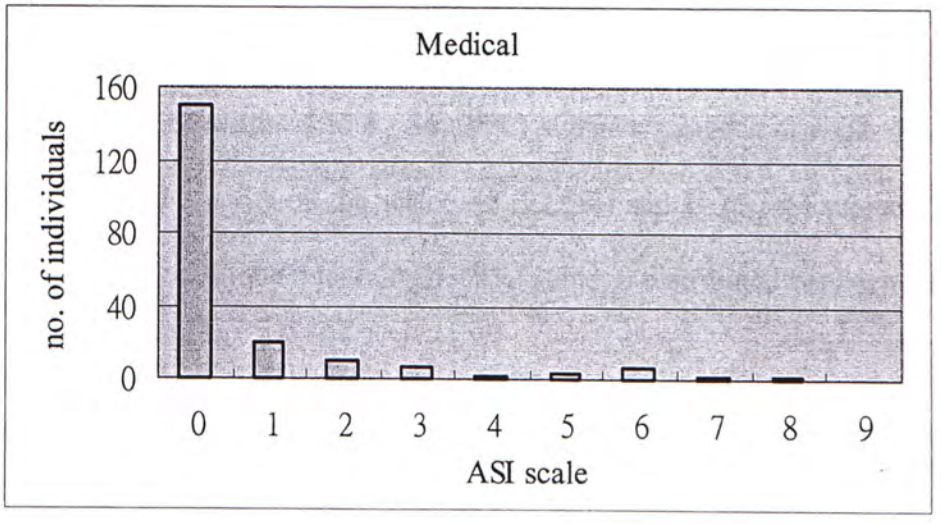

Year of Heroin Use
no. of individuals
60
40
20
0
0-5
6-10
11-15
16-20
21-25
26-30
31-35
Year

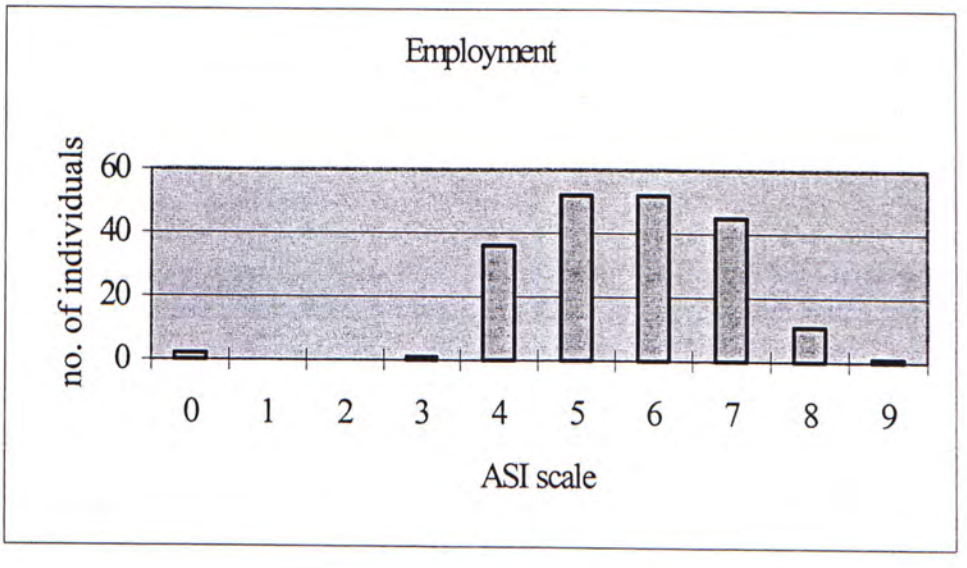
Fig. 4a) Distribution of ASI - Medical of 200 heroin-dependent subjects.

4b) Distribution of ASI - Employment in heroin-dependent subjects.

Fig. 4a) ASI - Medical



b) ASI - Employment



3.2.2 ASI – Employment

Fig. 4b showed the ASI distribution of employment of the 200 heroin-dependent subjects to be within 4 to 8. 36 (18%) subjects scored a 4, while 52 subjects (26%) each scored 5 and 6 on the scale. 45 (22.5%) and 11 (5.5%) subjects scored 7 and 8 respectively with the remaining 4 (2%) subjects distributed between 0 to 3 and 9.

3.2.3 ASI – Drug

Fig. 4c shows that the heroin-dependent subjects were distributed across the scale of 0 to 9. 43 (21.5%) subjects scored 5, 25 (12.5%), 28 (14%), 37 (18.5%) and 24 (12%) subjects scored 2, 3, 4 and 6 respectively. The remaining 43 (21.5%) subjects were distributed at index 0 - 1 and 7 - 9. Taken together, a total of 78.5% of subjects scored between 2 and 6 for drug.

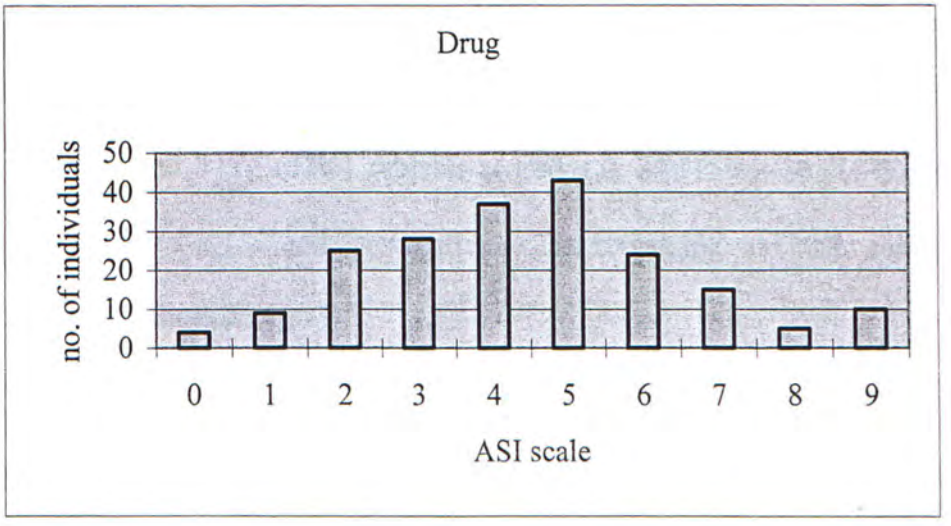
3.2.4 ASI – Legal

Fig. 4d shows that 53 (26.5%) of heroin-dependent subjects had an index of 0. 23 (11.5%), 35 (17.5%) and 22 (11%) subjects scored 1, 2 and 3 respectively. 31 (15.5%) individuals scored between 4 to 6 and 36 (18%) subjects scored between 7 to 9. This indicated that more than half of the subjects had ASI from 0 - 3.

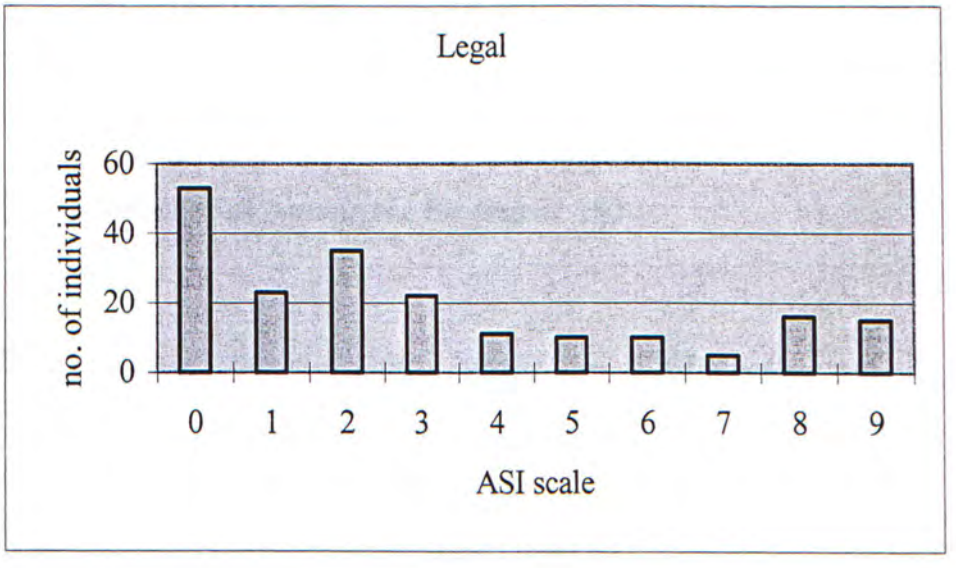
Fig. 4c) Distribution of ASI - Drug of 200 heroin-dependent subjects.

4d) Distribution of ASI - Legal in heroin-dependent subjects.

c) ASI – Drug



d) ASI - Legal



3.2.5 ASI – Family/Social Relationships

Fig.4e shows that the ASI scores of the heroin-dependent subjects were mostly found between 0 and 3. 74 (37%) subjects scored a 0, 30 (15%), 36 (18%) and 20 (10%) subjects scored a 1, 2 and 3 respectively. The remaining 40 (20%) subjects scored in the indices ranges of 4 to 9.

3.2.6 ASI – Psychiatry

Fig. 4f shows that 86 (43%) subjects scored an index of 0. 33 (16.5%), 23 (11.5%) and 22 (11%) subjects scored 1, 2 and 3 respectively. The remainding 36 (18%) scored between 4 and 7 with only 2 (1%) of these subjects scoring 7. No subjects scored 8 or 9. Taken together, 82% of subjects scored between 0 and 3.

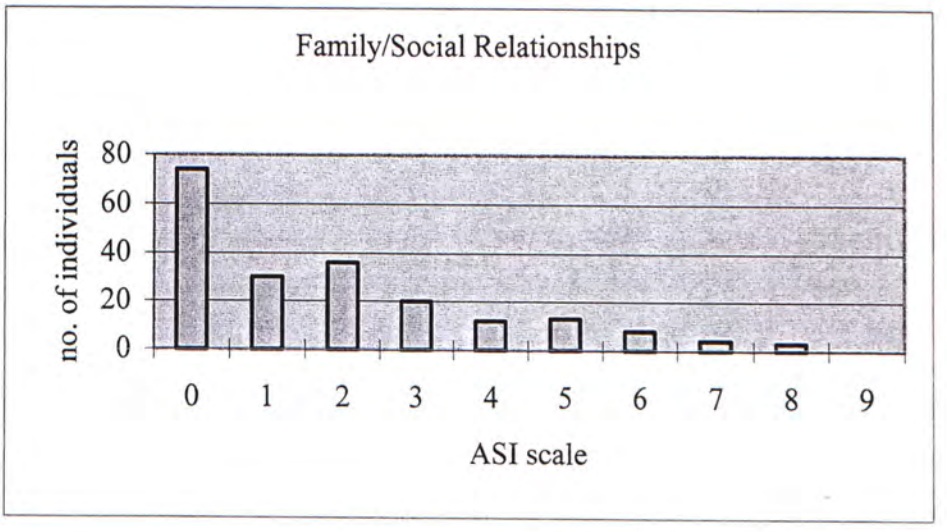
3.2.7 Correlations Among the Factors of ASI

Table 1 showed the correlations among the 6 ASI factors that were examined. Pearson's correlation showed statistically significant correlations between medical and family/social relationships status ($p=0.004$, $r=0.192$), medical and psychiatric status ($p=0.002$, $r=0.244$), employment and psychiatric status ($p=0.000$, $r=0.274$), drug and legal status ($p=0.000$, $r=0.381$) as well as family/social relationships and psychiatric status ($p=0.000$, $r=0.465$).

Fig. 4e) Distribution of ASI – Social relationships of 200 heroin-dependent subjects.

4f) Distribution of ASI - Psychiatry of 200 heroin-dependent subjects.

e) ASI – Family/Social Relationships



f) ASI – Psychiatry

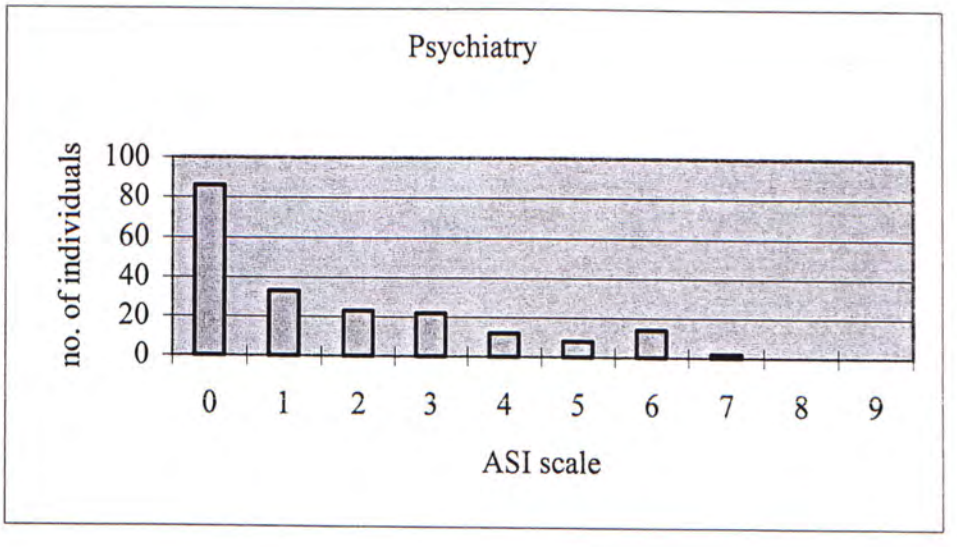


Table 1 Correlation between the 6 ASI factors. Pearson correlation coefficients (r) are shown in the boxes. * indicates a statistical significance level at $p<0.05$.

Table 1

	Medical	Employment	Drug	Legal	Family/Social Relationships	Psychiatry
Medical	1					
Employment	0.111	1				
Drug	0.076	0.017	1			
Legal	0.001	0.072	*0.381	1		
Family/Social Relationships	*0.192	0.181	-0.019	-0.046	1	
Psychiatry	*0.244	*0.274	0.036	-0.087	*0.465	1

Pearson correlation coefficient (r)

3.3 A118G Polymorphism in Exon 1 of the Human Mu Opioid Receptor (hMOR) Gene

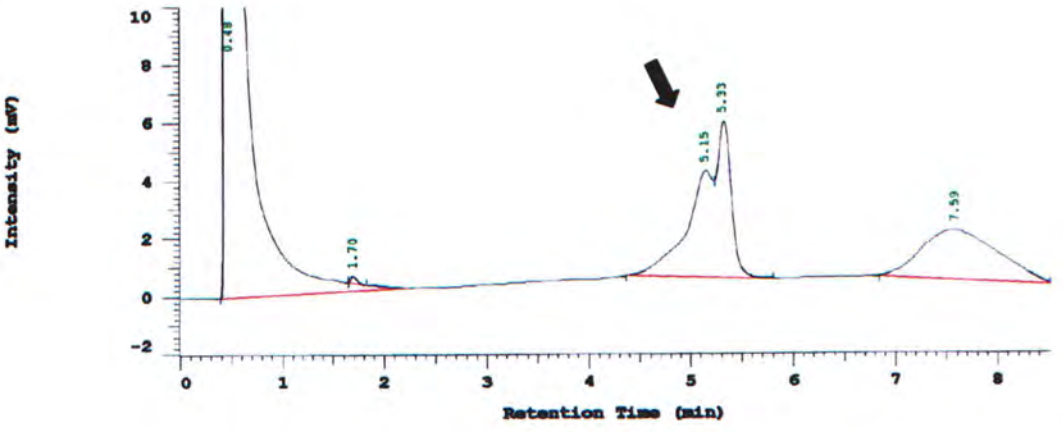
PCR products of exon 1 of the hMOR gene containing the A118G polymorphism were analyzed by dHPLC. The chromatogram of a heterozygous AG individual shows the presence of two well-resolved peaks (heteroduplex) (Fig. 5a). This was confirmed by direct sequencing (Fig. 5b). The polymorphic site is indicated by an arrow. An individual with homozygous AA or GG is characterized by the presence of a single peak in the chromatogram as observed in Figs. 6 and 7 respectively. In order to distinguish whether the single peak represents a homozygous AA or homozygous GG individual, each sample was mixed with a control DNA containing homozygous AA. If two well-resolved peaks (Fig. 8a) results from this mixture, the individual is homozygous GG due to the formation of a heteroduplex similar to that observed in heterozygous AG individuals. If a single peak results after the addition of the control DNA, the individual is a homozygous AA (Fig. 9a). Direct sequencing of the DNA gave a 100 % match to the results obtained from the dHPLC (Figs. 8b & 9b). The homozygous AA individual carried the sequence TGGC**A** while the homozygous GG individual carried the sequence TGGC**G** as indicated by the arrows (Fig.8b & 9b). A heterozygote individual carries both **A** and **G**.

The distributions of genotype and allele frequencies of the A118G polymorphism of the hMOR gene in the control and heroin-dependent subjects are shown in Tables 2a and 2b respectively. Table 2a showed that, out of the 97 control subjects, 51 (52.6%) of them carried the AA homozygous genotype, 11 (11.3%) carried the GG

Fig. 5a) Chromatogram of the A118G polymorphism of a heterozygous AG individual. Two well-resolved peaks are shown (indicated by arrow).

b) The pattern of direct sequencing for the A118G polymorphism of a heterozygous AG individual. The arrow indicates the polymorphic site.

Fig. 5a)


Intensity (mV)
10
8
6
4
2
0
-2
0.48
1.70
5.15
5.33
7.59
0
1
2
3
4
5
6
7
8
Retention Time (min)

b)

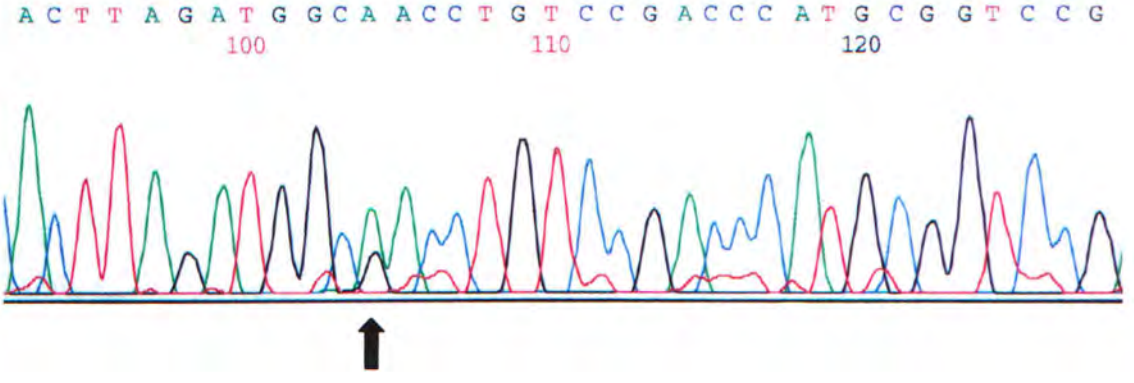

A C T T A G A T G G C A A C C T G T C C G A C C C A T G C G G T C C G
100
110
120

Fig. 6 Chromatogram of the A118G polymorphism of a homozygous AA individual. A single peak is shown (indicated by arrow).

Fig. 7 Chromatogram of the A118G polymorphism of a homozygous GG individual. A single peak was also observed (indicated by arrow).

Fig. 6

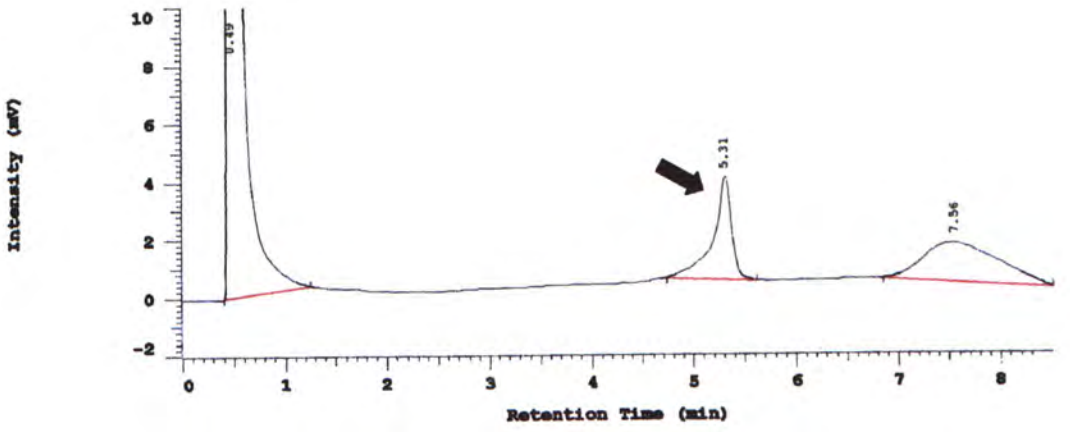

Intensity (mV)
10
8
6
4
2
0
-2
0.49
5.31
7.56
0
1
2
3
4
5
6
7
8
Retention Time (min)

Fig. 7

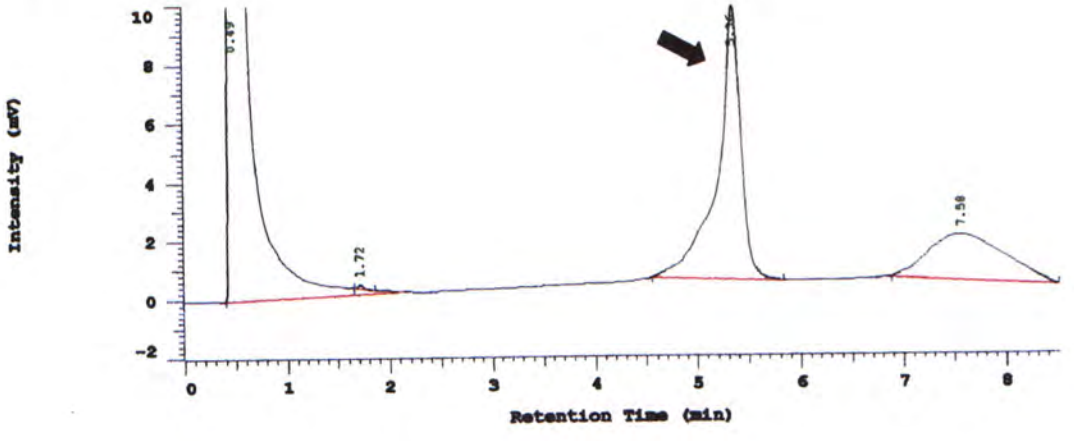
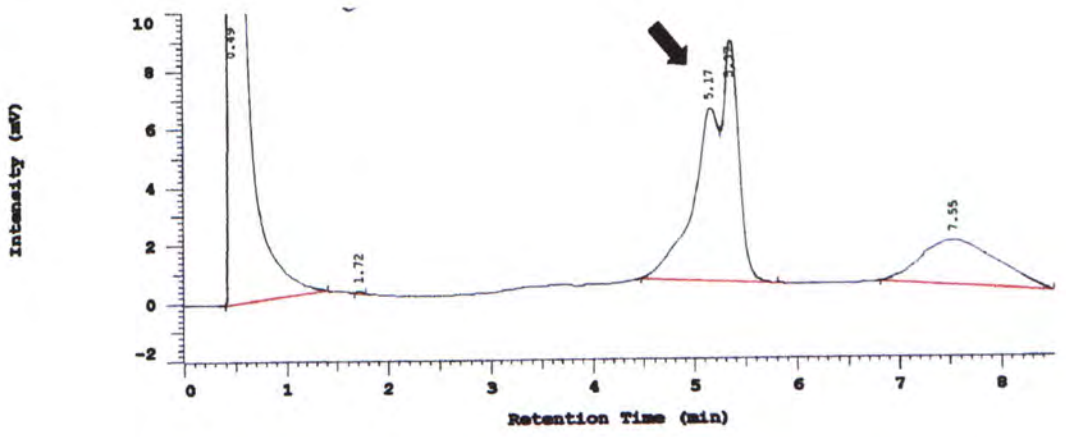

Intensity (mV)
10
8
6
4
2
0
-2
0.49
1.72
7.58
0
1
2
3
4
5
6
7
8
Retention Time (min)

Fig. 8a) Chromatogram of the A118G polymorphism of a homozygous GG individual. Two well-resolved peaks are shown (indicated by arrow) when control DNA (homozygous AA) was mixed with this sample.

b) The pattern of direct sequencing for the A118G polymorphism of the homozygous GG individual. The arrow indicates the polymorphic site.

Fig. 8a)


Intensity (mV)
10
8
6
4
2
0
-2
0
1
2
3
4
5
6
7
8
Retention Time (min)
1.72
5.17
7.55

b)

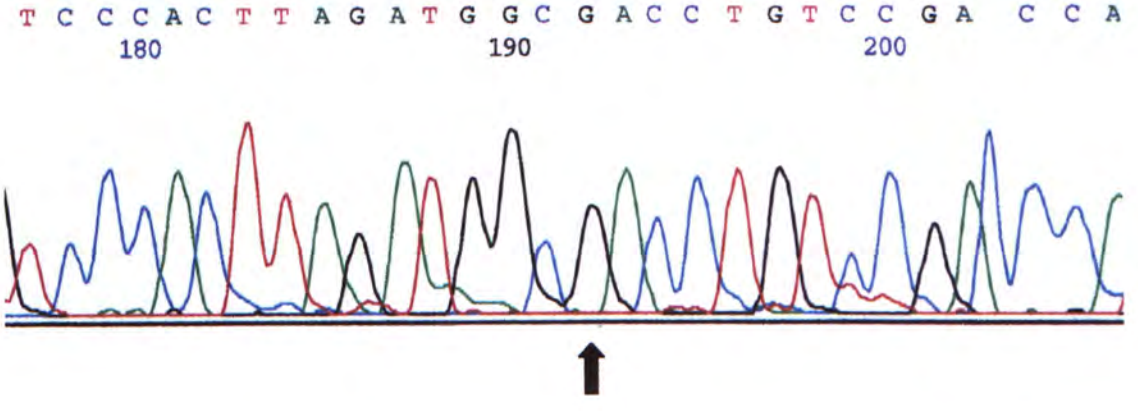
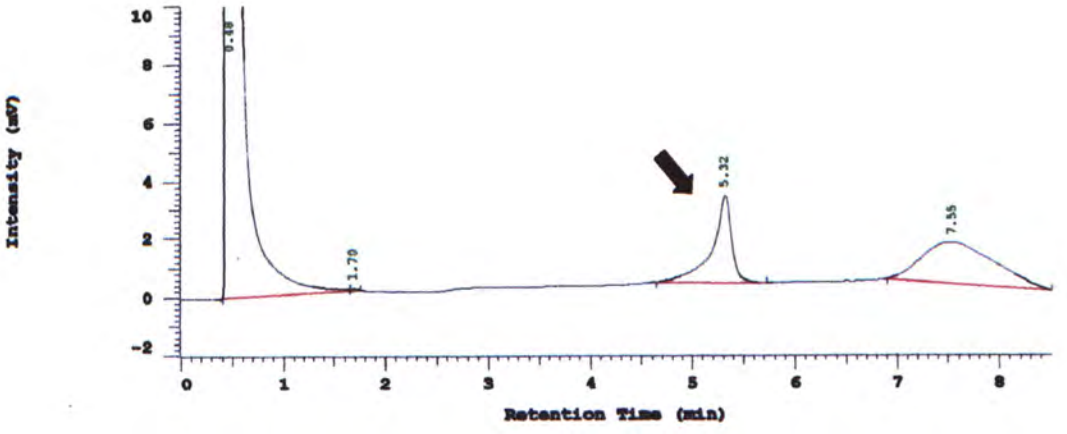

T C C C A C T T A G A T G G C G A C C T G T C C G A C C A
180
190
200

Fig. 9a) Chromatogram of the A118G polymorphism of a homozygous AA individual. A single peak (indicated by arrow) was observed after mixing the sample with control DNA (homozygous AA).

b) Direct sequencing pattern for the A118G polymorphism of a homozygous AA individual. The arrow indicates the polymorphic site.

Fig. 9a)


Intensity (mV)
10
8
6
4
2
0
-2
0.48
1.79
5.32
7.35
0
1
2
3
4
5
6
7
8
Retention Time (min)

b)

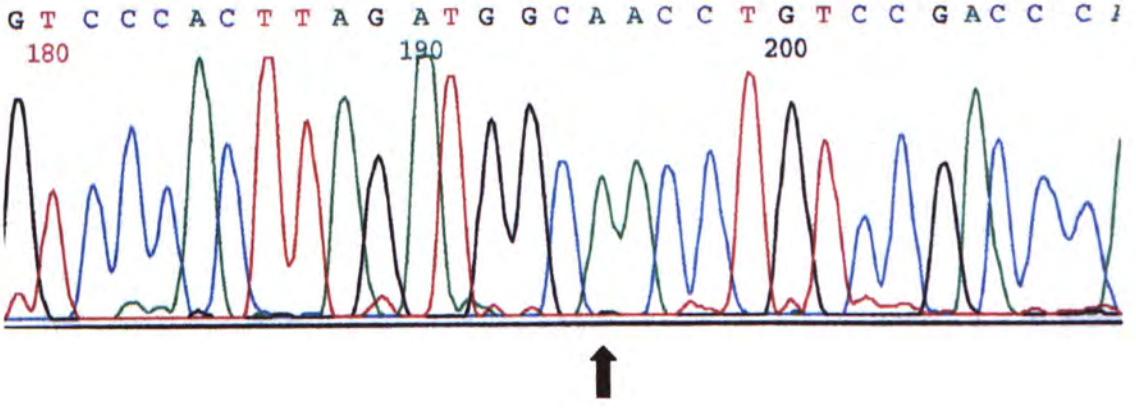

G T C C C A C T T A G A T G G C A A C C T G T C C G A C C C
180
190
200

Table 2a) Distribution of genotype frequency of the A118G polymorphism of the hMOR gene in control and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=6.176; df=2; p=0.046 for the comparison of heroin-dependent subjects and controls.

b) Allelic frequencies of A and G of the A118G polymorphism of the hMOR gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=5.792; df=1; p=0.016 for comparison of heroin-dependent subjects and controls.

Table 2a

	Genotype			Total
	AA	AG	GG	
Controls	51 (52.6%)	35 (36.1%)	11 (11.3%)	97
Heroin -dependent subjects	75 (37.5%)	92 (46.0%)	33 (16.5%)	200

2b)

	Allelic frequency		Total
	A	G	
Controls	137 (70.6%)	57 (29.4%)	194
Heroin-dependent subjects	242 (60.5%)	158 (39.5%)	400

homozygous genotype and 35 (36.1%) carried the heterozygous AG genotype. In comparison, of the 200 heroin-dependent subjects examined, 75 (37.5%) and 33 (16.5%) of the subjects had homozygous genotype AA and GG respectively. 92 (46%) of the heroin-dependent subjects were AG heterozygous carriers. Yates χ^2 analysis between the control and heroin-dependent subjects showed a significant difference ($\chi^2_{(2)}$=6.176; p=0.046) between the two groups.

When the allelic frequencies was examined, allele G was observed in a larger proportion of heroin-dependent subjects than in the controls (39.5% vs. 29.4%; Table 2b). The χ^2 test yielded a significant difference ($\chi^2_{(1)}$=5.792; p=0.016) between the two groups. The higher proportion of G was shown to be due to an increase in the number of GG homozygotes (16.5% in the heroin-dependent subjects vs. 11.3% in the control population.). This difference was shown earlier to be statistically significant (p=0.046; Table 2a). Notably, the proportion of heterozygotes among the heroin-dependent subjects was higher than that in the control population. Moreover the increase in GG homozygotes was accompanied by a decrease in AA homozygotes, this means the GG genotype is more common than the AA genotype in heroin-dependent subjects than in controls. Furthermore, it was noted that the prevalence of the A allele was higher in the control group than in the heroin-dependent group (70.6% vs. 60.5%, see Table 2b).

3.4 C1031G Polymorphism in Intron 2 of the hMOR Gene

The PCR product of 145bp size was subjected to *Hinf*I digestion and the resulting fragments were resolved on a 4% agarose gel that was stained with ethidium bromide. Genotype CC was represented by the presence of a single 124 bp band, whereas the genotype GG was represented by the presence of a 145 bp band. Both 124 bp and 145 bp bands can be found in the heterozygote CG individual (Fig. 10). The results of genotyping were confirmed by direct sequencing (Figs. 11a-c). A homozygous CC individual carries the sequence TAAAAAT**C** while a homozygous GG individual carries sequence TAAAAAT**G** as indicated by the arrows. A heterozygote individual carries both **C** and **G**.

Tables 3a and 3b show the C1031G polymorphism in intron 2 of the hMOR gene. Table 3a reveals that 64 of the controls (66%) carried the CC homozygous genotype, 8 controls (8.2%) carried the GG homozygous genotype and 25 (25.8%) were carriers of the CG genotype. In contrast, it was found that there were 108 (54%) and 31 (15.5%) heroin-dependent subjects with CC and GG homozygous genotype respectively. 61 heroin-dependent subjects (30.5%) were CG heterozygous carriers (Table 3a). Yates χ^2 analysis showed that $\chi^2_{(2)}$ =4.739; p=0.094. (NS)

Table 3b shows the allelic frequencies of C and G alleles. The C allele was the most common allele in both controls (78.9%) and heroin-dependent subjects (69.3%). However, the frequency of the G allele was found to be significantly higher ($\chi^2_{(1)}$=4.407; p=0.014) in the heroin-dependent subjects (30.8%) than in the controls

Fig. 10 A 145bp PCR product of the C1031G polymorphism of the hMOR gene was cut by the restriction enzyme *Hinf*I. A DNA marker (1kb DNA ladder) was loaded on the left.

Fig. 10

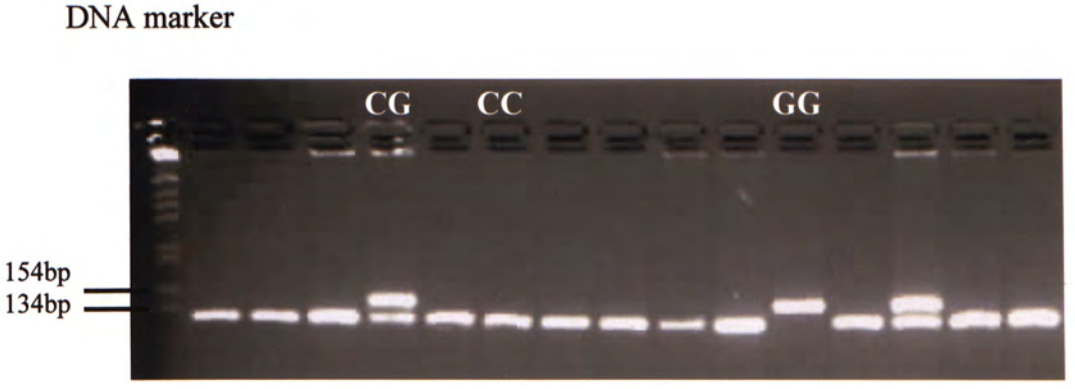
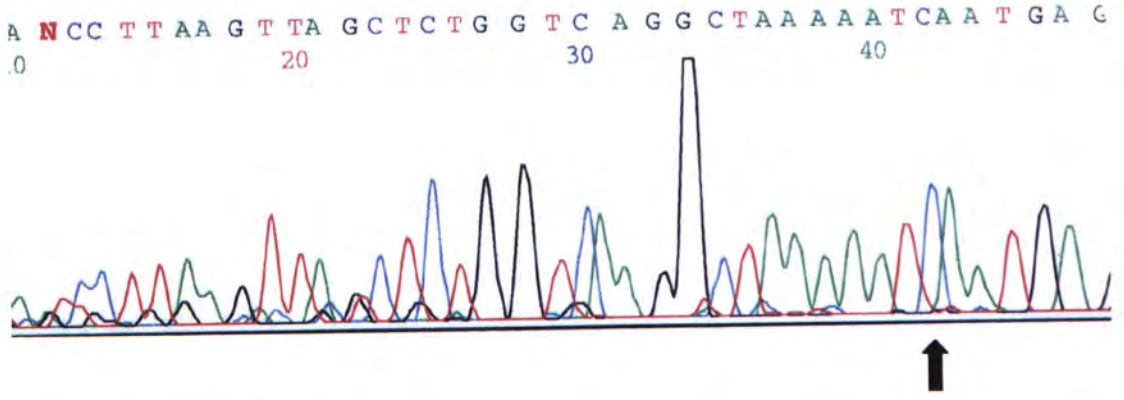


Fig. 11a) The pattern of direct sequencing the C1031G polymorphism of the hMOR gene of a homozygous CC individual. The arrow indicates the polymorphic site.

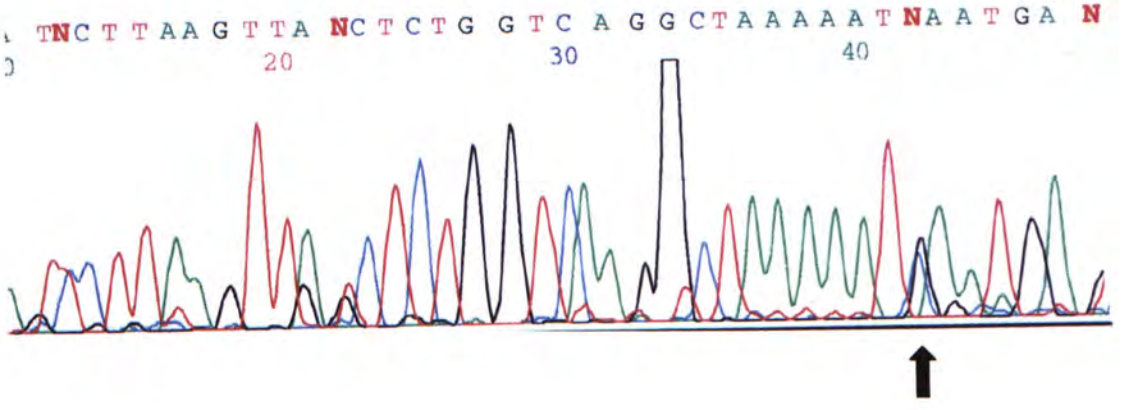
b) The pattern of direct sequencing the C1031G polymorphism of the hMOR gene of a heterozygous CG individual. The arrow indicates the polymorphic site.

c) The pattern of direct sequencing the C1031G polymorphism of the hMOR gene of a homozygous GG individual. The arrow indicates the polymorphic site.

Fig. 11a)


A NCC T T AA G T TA GC T C T G G T C A G G C T A A A A A T C A A T GA G
0
20
30
40

b)


TNC T T AA G T TA NC T C T G G T C A G G C T A A A A A T NA A T GA N
20
30
40

c)

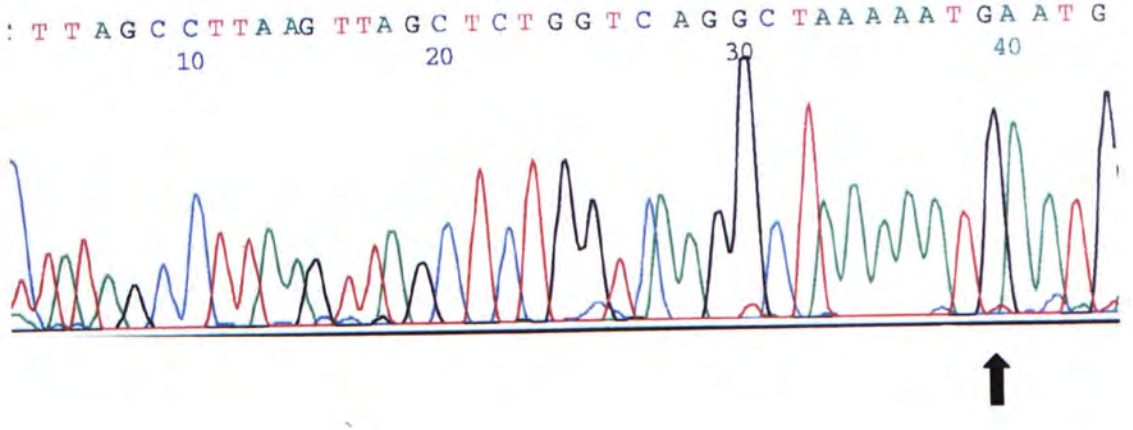

T T AG C C T T A AG T T A G C T C T G G T C A G G C T A A A A A T GA A T G
10
20
30
40

Table 3a) Genotype frequencies of the C1031G polymorphism of the hMOR gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=4.739; df=2; p=0.094 when heroin-dependent subjects were compared to the controls.

b) Allelic frequencies of C and G of the C1031G polymorphism of the hMOR gene in controls and heroin dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=4.407; df=1; p=0.014 when heroin-dependent subjects were compared to the controls.

Table 3a)

	Genotype			Total
	CC	CG	GG	
Controls	64 (66.0%)	25 (25.8%)	8 (8.3%)	97
Heroin-dependent subjects	108 (54.0%)	61 (30.5%)	31 (15.5%)	200

b)

	Allelic frequency		Total
	C	G	
Controls	153 (78.9%)	41 (21.1%)	194
Heroin-dependent subjects	277 (69.3%)	123 (30.7%)	400

(21.1%). It can be seen that the higher frequency of the G allele contributed to a two-fold increase in the number of homozygous GG (15.5%) among the heroin-dependent subjects as compared with the controls (8.3%) (Table 3a). Furthermore, the high G allele frequency also contributed to the higher percentage of heterozygous CG observed in the heroin-dependent subjects (Table 3a). There was a concomitant decrease in the number of homozygous CC individuals in the heroin-dependent group as the number of heterozygous CG and homozygous GG individuals increased.

3.5 T921C Polymorphism in the Exon 3 of the Human Delta Opioid Receptor (hDOR) Gene

The PCR product of 106bp size was subjected to *BstE*II digestion and the resulting fragments were resolved on a 4% agarose gel that was stained with ethidium bromide. The results of the genotyping were confirmed by direct sequencing (Figs. 13a-c). A homozygous TT individual carries the sequence TGGG**T**T while a homozygous GG individual carries the sequence TGGG**C**T as indicated by the arrows. A heterozygote carries both **T** and **C**.

Table 4 shows the T921C polymorphism in the exon 3 of the hDOR gene. Table 4a reveals that 59 controls (60.8%) carried the TT homozygous genotype, 4 controls (4.1%) carried the CC homozygous genotype and 34 (35.1%) were carriers of the TC heterozygote genotype. In the heroin-dependent group, it was found that there were 90 (45%) and 11 subjects (5.5%) with TT and CC homozygous genotype respectively. 99

Fig. 12 A 106bp PCR product of the T921C polymorphism of the hDOR gene was cut by the restriction enzyme *BStE*II. A DNA marker (1kb DNA ladder) was loaded on the left.

Fig. 12

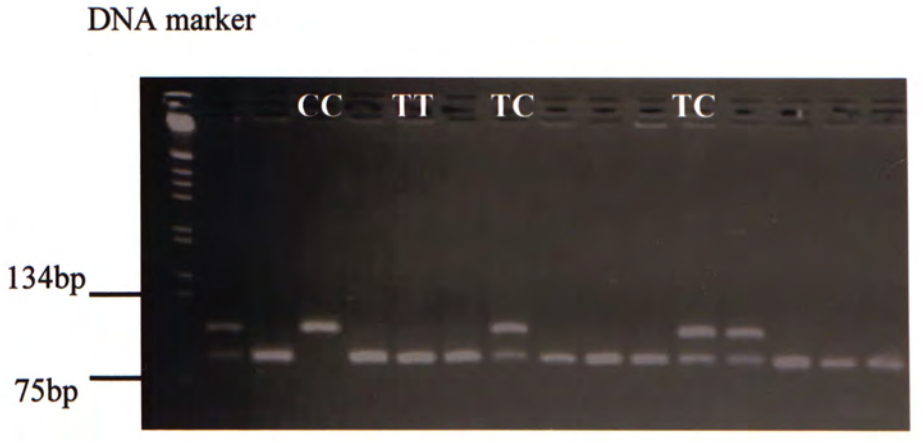
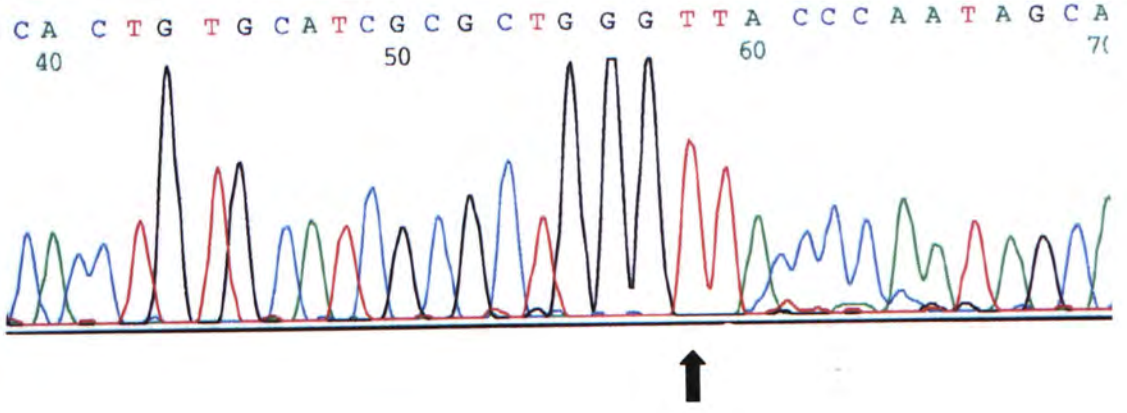


Fig. 13a) The pattern of direct sequencing the T921C polymorphism of the hDOR gene of a homozygous TT individual. The arrow indicates the polymorphic site.

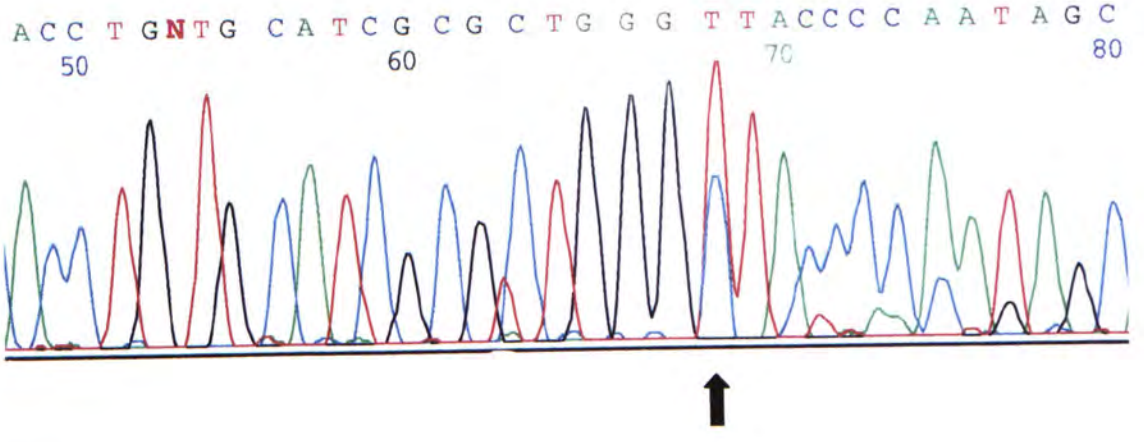
b) The pattern of direct sequencing the T921C polymorphism of the hDOR gene of a heterozygous TC individual. The arrow indicates the polymorphic site.

c) The pattern of direct sequencing the T921C polymorphism of the hDOR gene of a homozygous CC individual. The arrow indicates the polymorphic site.

Fig. 13a)


CA CTG TGCATCGCGCTGGGTTA CCCAATAGCA
40
50
60

b)


ACC TGNTG CATCG CGCTGGG TTACCCC AATAGC
50
60
70
80

c)

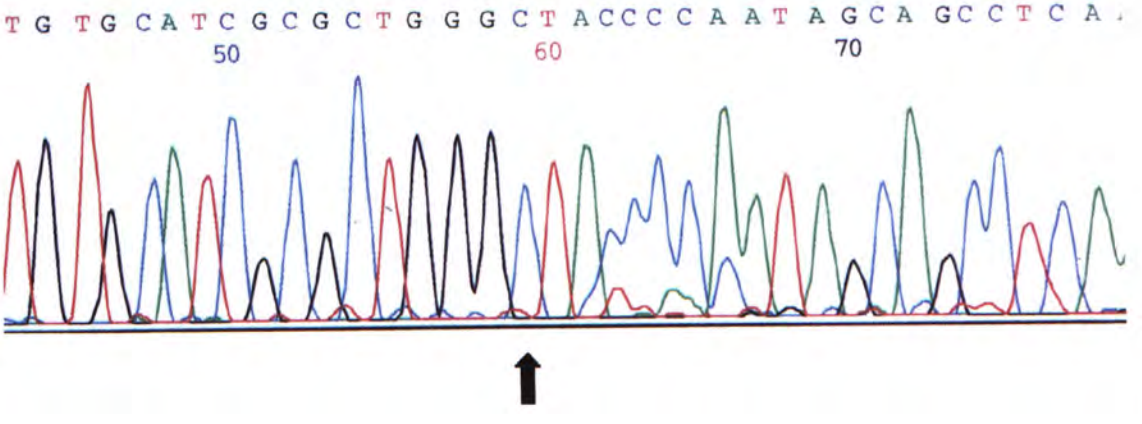

TG TG CATCGCGCTGGGCTACCCCAATAGCA GCCTCA
50
60
70

Table 4a) Distribution of genotype frequency of the T921C polymorphism of the hDOR gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=6.797; df=2; p=0.038 for comparison of heroin-dependent subjects and controls.

b) Allelic frequencies of T and C of the T921C polymorphism of the hDOR gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=4.853; df=1; p=0.028 for comparison of heroin-dependent subjects and controls.

Table 4a)

	Genotype			Total
	TT	TC	CC	
Controls	59 (60.8%)	34 (35.1%)	4 (4.1%)	97
Heroin-dependent subjects	90 (45.0%)	99 (49.5%)	11 (5.5%)	200

b)

	Allelic frequency		Total
	T	C	
Controls	152 (78.4%)	42 (21.6%)	194
Heroin-dependent subjects	279 (69.8%)	121 (30.3%)	400

heroin-dependent subjects (49.5%) were TC heterozygous (Table 4a). Yates χ^2 analysis showed that $\chi^2_{(2)}=6.797$; p=0.038.

Table 4b shows the allelic frequencies of the T and C alleles. The T allele was the most common allele in both controls (78.4%) and heroin-dependent subjects (69.8%). The prevalence of the T allele in the control group was significantly higher ($\chi^2_{(1)}=4.853$; p=0.028.) than that of the heroin-dependent group, whilst a higher prevalence of the C allele was found in the heroin-dependent group (30.3%) than in the control group (21.6%). It can be noted that the higher frequency of the T allele in the control group contributed to the major genotype, homozygous TT (60.8%) and heterozygous TC group (35.1%). The number of homozygote TT individuals was almost doubled that of TC heterozygotes (59 vs. 34; Table 4a). The relatively high percentage of heterozygous TC subjects in the heroin-dependent group contributed to the high C allele frequency (49.5% vs. 35.1%, Table 4a). In the heroin-dependent group there was a decrease in the number of homozygous TT individuals as the number of heterozygous TC and homozygous CC individuals increased.

3.6 Interaction Between Genotypes

3.6.1 Combined Genotypes of A118G and C1031G Polymorphisms of hMOR Gene

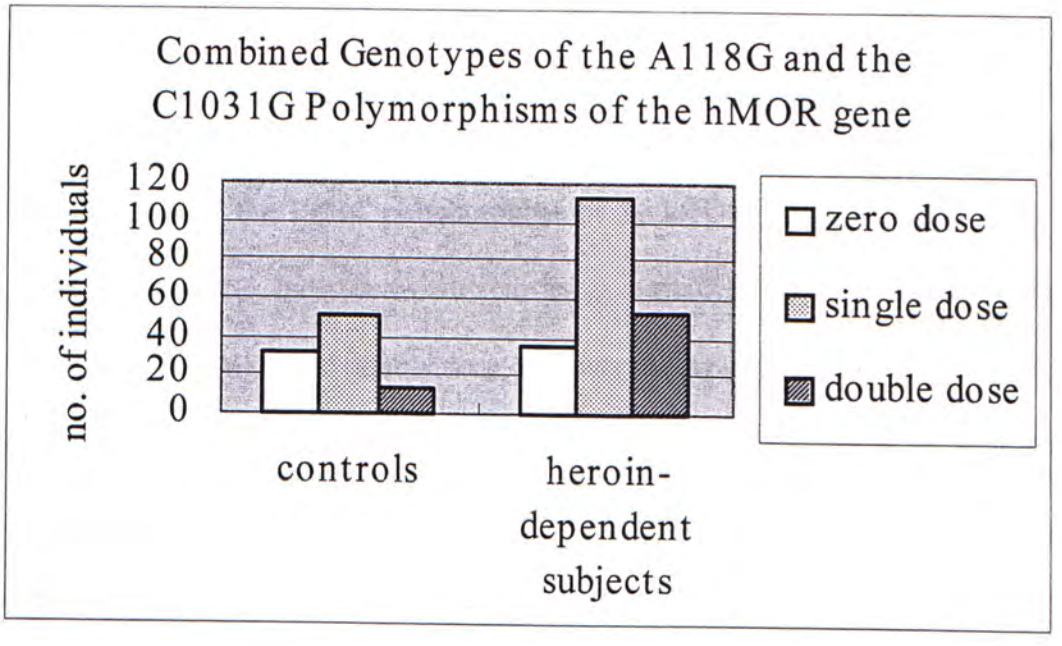
Fig 14 shows the genotype distribution of the combined A118G and C1031G polymorphisms of the hMOR gene in the control and heroin-dependent subjects.

Fig. 14 Combined genotypes of the A118G and the C1031G polymorphisms of the hMOR gene in control and heroin-dependent subjects. Subjects who had the AA of A118G and CC of C1031G were scored as 0, this means they had a zero dose of the susceptibility gene. Subjects who had the AA, AG of A118G or the CC, CG of C1031G were labeled as 1, this means they had a single dose of the susceptibility gene. Subjects who carried the AG, GG of A118G and CG, GG of C1031G were scored as 2, this indicates that they had a double dose of the susceptibility gene.

Fig. 14a) Distribution of combined genotypes of the A118G and the C1031G polymorphisms of the hMOR gene in controls and heroin-dependent subjects.

Groups	Genotype		
	0	1	2
Controls (n=97)	32	51	14
Heroin-dependent subjects (n=200)	35	113	52

b) Bar-chart representation of **a)**



Individuals who carried the 0, 1 or 2 genotype corresponded to those who had zero, single or double dose of the susceptibility genotypes. Subjects with the 1 vs 0, 1 vs 2 and 2 vs 0 genotypes were compared. χ^2 tests showed statistical significance in 1 vs 0 and 2 vs 0 genotypes. However, the highest relative odds (RO) were found when the 2 vs 0 genotypes were compared between controls and heroin- dependent subjects (RO=3.396, p=0.001; see Table 5a).

3.6.2 Combined Genotypes of A118G Polymorphism of the hMOR Gene and the T921C Polymorphism of the hDOR Gene

Fig 15 shows the genotype distribution of the combined A118G polymorphism of the hMOR gene and the T921C polymorphism of the hDOR gene in controls and heroin-dependent subjects. Individuals who carried 0, 1 and 2 genotypes correspond to those who had zero, single and double dose of susceptibility genotypes. Subjects with the 1 vs 0, 1 vs 2 and 2 vs 0 susceptibility genotypes were compared. χ^2 tests showed statistical significance in the 1 vs 2 and 2 vs 0 genotypes. The highest relative odds (RO) were found when the 2 vs 0 genotypes were compared between controls and heroin-dependent subjects (RO=3.434, p=0.0005; see Table 5b).

3.6.3 Combined Genotypes of the C1031G Polymorphism of the hMOR Gene and the T921C Polymorphism of the hDOR Gene

Fig 16 shows the genotype distribution of the combined C1031G polymorphism of the hMOR gene and the T921C polymorphism of the hDOR gene in controls and

Table 5 Relative Odds (RO) determined by logistic regression analysis of the combined effects of A118G, C1031G and T921C genotypes. Yates Chi-square test was used to compare the prevalence of these combined genotypes between the controls and heroin-dependent subjects. * Indicates statistical significance level at $p<0.05$.

Table 5

	Relative Odds	χ^2 value	P-value
A. Combined A118G and C1031G			
1 vs 0 genotypes	2.026	5.738	*0.017
1 vs 2 genotypes	1.676	2.268	0.132
2 vs 0 genotypes	3.396	10.358	*0.001
B. Combined A118G and T921C			
1 vs 0 genotypes	1.476	1.665	0.197
1 vs 2 genotypes	2.327	7.073	*0.008
2 vs 0 genotypes	3.434	11.970	*0.001
C. Combined C1031G and T921C			
1 vs 0 genotypes	1.967	5.824	*0.016
1 vs 2 genotypes	1.790	2.419	0.120
2 vs 0 genotypes	3.520	10.211	*0.001

Fig. 15 Combined genotype of the A118G polymorphism of the hMOR gene and the T921C polymorphism of the hDOR gene in control subjects and heroin-dependent subjects. Subjects who had the AA of A118G and TT of T921C were scored as 0, this means they had a zero dose of susceptibility gene. Subjects who had the AA, AG of A118G or the TC, TT of T921C were labeled as 1, this means they had a single dose of susceptibility gene. Subjects who carried the AG, GG of A118G and TC, CC of T921C were scored as 2, this indicates that they had a double dose of susceptibility gene.

Fig. 15a) Distribution of combined genotypes of the A118G polymorphism of the hMOR gene and the T921C polymorphism of the hDOR gene in controls and heroin-dependent subjects.

Groups	Genotype		
	0	1	2
Controls (n=97)	30	50	17
Heroin-dependent subjects (n=200)	37	91	72

b) Bar-chart representation of **a)**

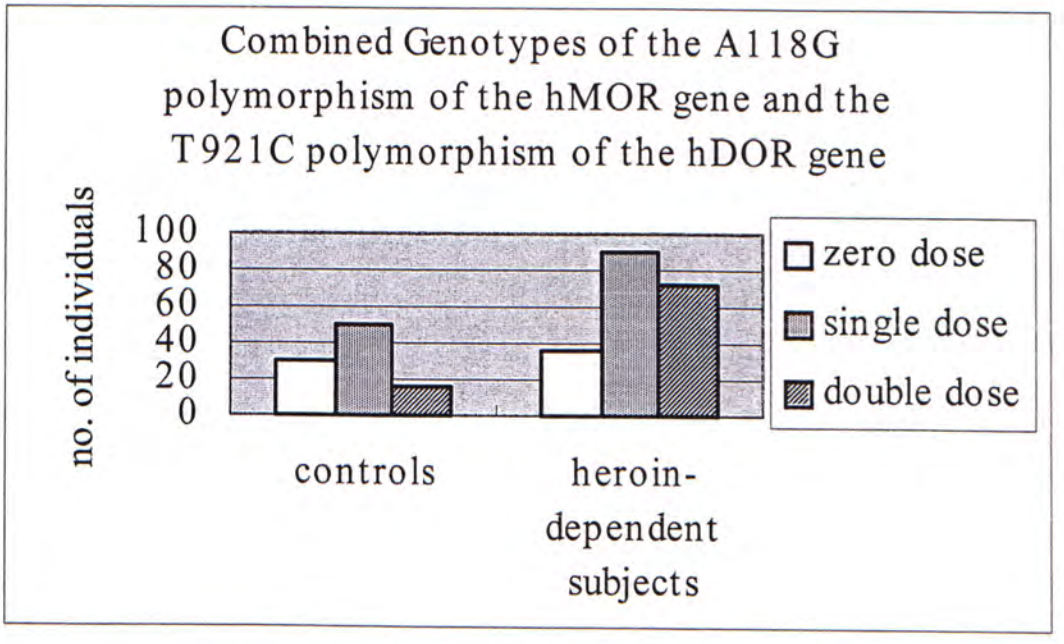
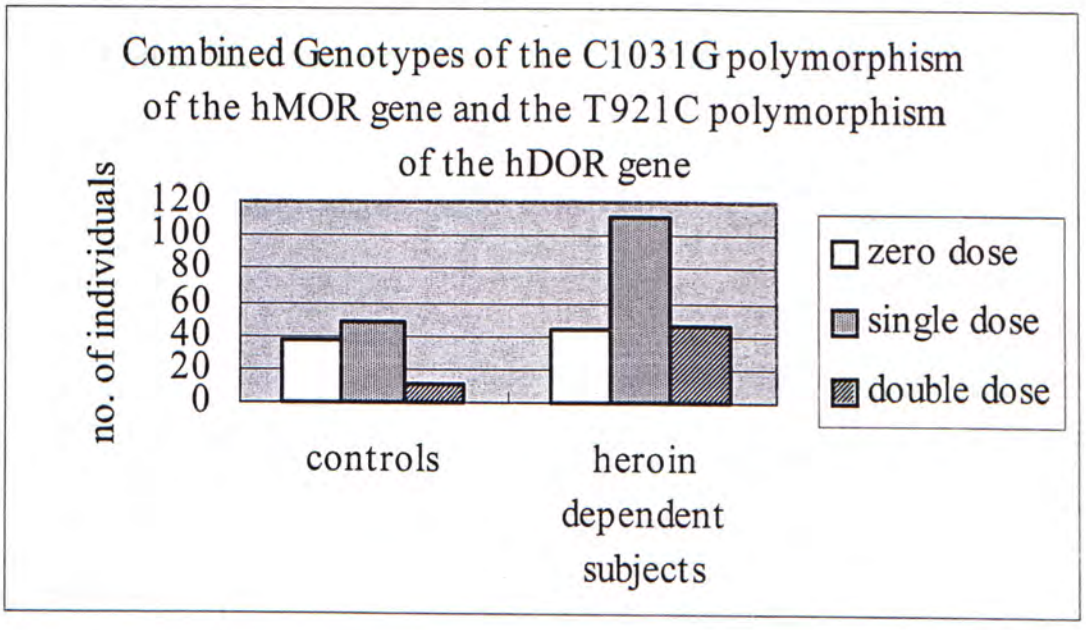


Fig. 16 Combined genotype of the C1031G polymorphism of the hMOR gene and the T921C polymorphism of the hDOR gene in control and heroin-dependent subjects. Subjects who had the CC of C1031G and TT of T921C were scored as 0, this means they had a zero dose of susceptibility gene. Subjects who had the CC, CG of C1031G or the TC, TT of T921C were labeled as 1, this means they had a single dose of susceptibility gene. Subjects who carried the CG, GG of C1031G and TC, CC of T921C were scored as 2, this indicates that they had a double dose of susceptibility gene.

Fig. 16a) Distribution of combined genotypes of the C1031G polymorphism of the hMOR gene and the T921C polymorphism of the hDOR gene in controls and heroin-dependent subjects.

Groups	Genotype		
	0	1	2
Controls (n=97)	37	49	11
Heroin-dependent subjects (n=200)	43	112	45

b) Bar-chart representation of **a)**



heroin-dependent subjects. Individuals who carried 0, 1 and 2 genotypes corresponded to those who had zero, single and double dose of susceptibility genotypes. Subjects with the 1 vs 0, 1 vs 2 and 2 vs 0 susceptibility genotypes were compared. χ^2 tests showed statistical significance in 1 vs 0 and 2 vs 0 genotypes. The highest relative odds (RO) were found when the 2 vs 0 genotypes were compared between controls and heroin addicts (RO=3.520, p=0.001; see Table 5c).

3.7 Correlation Between Allelic Frequencies and Factors of the ASI

Table 6 shows the correlation between allelic frequencies and the different factors such as medical, employment, drug, legal, family/social relationships and psychiatry of the ASI. χ^2 tests showed statistically significant correlations between allelic frequencies and ASI factors between the G allele of the C1031G polymorphism and family/social relationships status (p=0.00005) as well as psychiatric status (p=0.0068).

3.8 3' VNTR Polymorphism of the DAT Gene

From Table 7a, it can be observed that the 10-repeat allele occurs most frequently in the Hong Kong population. The next most common one is the 9-repeat allele. The 9-repeat allele can be revealed by the presence of a 440bp band. The 6, 7, 10 and 11-repeat allele were indicated by the presence of 320bp, 360 bp, 480bp and 520bp bands respectively (Fig 17). The genotype frequencies of 3' VNTR of the DAT gene were: 1 with genotype 6/6, 2 with 9/9, 5 with each of 10/7and 11/10, 33 with 10/9, 198 with

Table 6 Correlation between allelic frequencies and factors of ASI. Subjects scored ASI indices between 0 - 4 or between 5 - 9 are regarded as less severe or more severe respectively.

Table 6

	P-value	df
A118G of hMOR (allele A vs allele G) **less severe subjects compared to more severe subjects**		
Medical	0.195	1
Employment	0.666	1
Drug	0.295	1
Legal	0.887	1
Family/Social relationships	0.755	1
Psychiatry	0.078	1
C1031G of hMOR (allele C vs allele G) **less severe subjects compared to more severe subjects**		
Medical	0.639	1
Employment	0.180	1
Drug	0.067	1
Legal	0.057	1
Family/Social relationships	*0.00005	1
Psychiatry	*0.0068	1
T921C of hDOR (allele T vs allele C) **less severe subjects compared to more severe subjects**		
Medical	0.744	1
Employment	0.818	1
Drug	0.266	1
Legal	0.533	1
Family/Social relationships	0.757	1
Psychiatry	0.616	1
3' VNTR of DAT (allele 9 vs allele other than 9) **less severe subjects compared to more severe subjects**		
Medical	0.133	1
Employment	0.143	1
Drug	0.299	1
Legal	0.397	1
Family/Social relationships	0.174	1
Psychiatry	0.829	1

***Taq*IA polymorphism of DRD2 (allele A1 vs allele A2) less severe subjects compared to more severe subjects**	P-value	df
Medical	0.422	1
Employment	0.319	1
Drug	0.175	1
Legal	0.276	1
Family/Social relationships	0.275	1
Psychiatry	0.283	1
***Nci*I polymorphism of GABRG2 (allele A vs allele G) less severe subjects compared to more severe subjects**		
Medical	0.675	1
Employment	0.576	1
Drug	0.425	1
Legal	0.639	1
Family/Social relationships	0.176	1
Psychiatry	0.236	1

Table 7a) Genotype frequencies of the 3' VNTR polymorphism of the DAT gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown.

b) Genotype frequencies of allele 9 of the 3' VNTR of the DAT gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=1.311; df=2; p=0.519 when heroin-dependent subjects were compared to the controls. * indicates an allele other than 9 of the DAT gene.

c) Allelic frequencies of the 9 allele and an allele other than 9 of the 3' VNTR of the DAT gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed that χ^2=0.871; df=1; p=0.351 when heroin-dependent subjects were compared with the controls. * indicates an allele other than 9 of the DAT gene.

Table 7a)

	Genotype						
	6/6	9/9	10/7	10/9	10/10	11/10	Total
Controls	0 (0%)	0 (0%)	2 (2.4%)	10 (12%)	68 (81.9%)	3 (3.6%)	83
Heroin-dependent subjects	1 (0.6%)	2 (1.2%)	3 (1.9%)	23 (14.3%)	130 (80.7%)	2 (1.2%)	161

b)

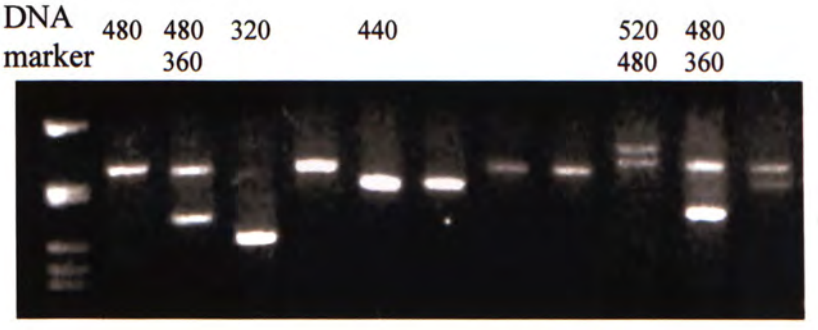
	Genotype			Total
	9/9	9/*	*/*	
Controls	0 (0%)	10 (12%)	73 (88%)	83
Heroin-dependent subjects	2 (1.2%)	23 (14.3%)	136 (84.5%	161

c)

	Allelic frequency (number of repeat)		Total
	9	*	
Controls	10 (6%)	156 (94%)	166
Heroin-dependent subjects	27 (8.4%)	295 (91.6%)	322

Fig. 17 Observed alleles ranged from 6 to 11 repeats (320-520bp). The 6/6, 9/9, 10/10 genotypes can be seen by the presence of the band with size 320bp, 440bp and 480bp respectively. 10/7, 10/9 and 10/11 genotypes can be identified by the band size 480bp and 360bp; 480bp and 440bp; 480bp and 520bp respectively.

Fig. 17



10/10. Collapsing the genotypes according to the presence of the 9-repeat allele (9/9 or 9/*) or absence of it (*/*), it may be seen (Table 7b) that, none of the controls (0%) carried the 9/9 homozygous genotype and 73 controls (88%) carried the */* homozygous genotype. The rest of them (12%) were carriers of the 9/* heterozygous genotype. In contrast, it was found that there were only 2 heroin-dependent subjects (1.2%) and 136 heroin-dependent subjects (84.5%) with 9/9 homozygous and */* homozygous genotype respectively. 23 heroin-dependent subjects (14.3%) were 9/* heterozygous carriers (see Table 7b).

From Table 7c, it was found that the percentage of carriers of the 9-repeat allele was not significantly different between the control and the heroin-dependent group (6% vs 5.4%). So was the distribution of a carrier with allele other than 9 in both groups (94% vs 91.6%). Therefore, the present study revealed no statistically significant difference between heroin-dependent subjects and controls in both genotype (p=0.519) and allelic frequencies (p=0.351) as shown in Table 6b and 6c.

3.9 *Taq*I A Polymorphism of the DRD2 Gene

From Table 8a, it may be seen that 11 controls (12.4%) carried the A1 homozygous genotype and 27 controls (30.3%) carried the A2 homozygous genotype. The remainder (57.3%) were carriers of the A1A2 genotype. In comparison, it was found that there were 28 (16.1%) and 56 (32.2%) heroin-dependent subjects having A1 homozygous and A2 homozygous genotypes respectively. 81 heroin-dependent subjects (51.7%) were A1A2 heterozygous carriers (see Table 8a).

Table 8a) Genotype frequencies of the *Taq*I A polymorphism of the DRD2 gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed χ^2=0.959; df=2; p=0.619 when heroin-dependent subjects were compared with the controls.

b) Allelic frequencies of A1 and A2 of *Taq*I A polymorphism of the DRD2 gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed χ^2=0.043; df=1; p=0.836 when heroin-dependent subjects were compared with the controls.

Table 8a)

	Genotype			Total
	A1A1	A1A2	A2A2	
Controls	11 (12.4%)	51 (57.3%)	27 (30.3%)	89
Heroin-dependent subjects	28 (16.1%)	90 (51.7%)	56 (32.2%)	174

b)

	Allelic frequency		Total
	A1	A2	
Controls	73 (41.0%)	105 (59.0%)	178
Heroin-dependent subjects	146 (42.0%)	202 (58.0%)	348

From Table 8b, it may be seen that the A2 allele was the most common allele in both controls (59%) and heroin-dependent subjects (58%). Moreover, a slightly higher prevalence of the A2 allele was found in the control group (59% vs 58%) as compared with the heroin-dependent group. It was proposed that the A1 allele maybe the potential allele for the contribution in predisposition of heroin dependence. However, there was no significant difference between the frequencies of A1 allele in the control (41%) and heroin-dependent group (42%)(p=0.836; Table 8b). It can also be observed that there was no statistically significant difference between heroin-dependent subjects and controls in the genotype frequency (p=0.619) as shown in Table 8a for the *Taq*I A polymorphism of the DRD2 gene.

3.10 *Nci*I Polymorphism of the GABRG2 Gene

The 287bp PCR product was subjected to *Nci*I digestion and the resulting fragments were resolved on a 5% agarose gel that was stained with ethidium bromide. Genotype AA was revealed by the presence of a single 287 bp band, whereas genotype GG was represented by the presence of a 263 bp band and the absence of 287 bp band. Both the 287 bp and 263 bp bands were found in the heterozygote AG sample (Fig. 18).

Table 9a shows that 1 control (1%) carried the AA homozygous genotype and 49 controls (51%) carried the GG homozygous genotype. The rest of the group (47.9%) carried the AG genotype. It was found that there were 12 heroin-dependent subjects (7.1%) and 97 heroin-dependent subjects (57.1%) with AA homozygous and GG homozygous genotype respectively. 71 heroin-dependent subjects (41.8%) were AG

Fig. 18 The 287 bp PCR product was subjected to *Nci*I digestion and the resulting fragments were resolved on a 5% agarose gel that was stained with ethidium bromide. A DNA marker (1 kb DNA ladder) was loaded on the left.

Fig. 18

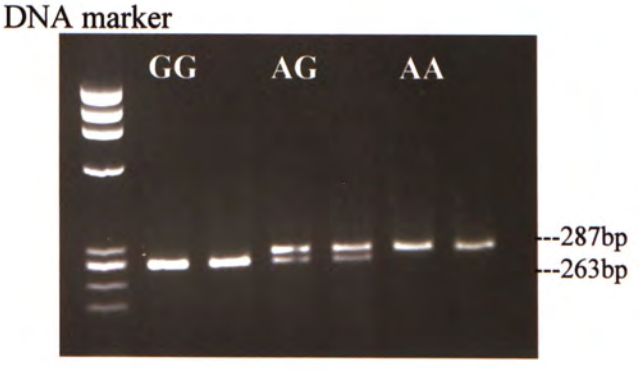


Table 9a) Genotype frequencies of the *Nci*I polymorphism of the GABRG2 gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed χ^2=5.073; df=2; p=0.079 when heroin-dependent subjects were compared with the controls.

b) Allelic frequencies of A and G of the *Nci*I polymorphism of the GABRG2 gene in controls and heroin-dependent subjects. The absolute number of cases and the percentage of total (in brackets) are shown. Yates χ^2 analysis showed χ^2=0.540; df=1; p=0.462 when heroin-dependent subjects were compared with the controls.

Table 9a)

	Genotype			Total
	AA	AG	GG	
Controls	1 (1.%)	46 (47.9%)	49 (51%)	96
Heroin-dependent subjects	12 (7.1%)	71 (41.8%)	87 (51.2%)	170

b)

	Allelic frequency		Total
	A	G	
Controls	48 (25%)	144 (75%)	192
Heroin-dependent subjects	95 (27.9%)	245 (72.1%	340

heterozygous carriers (see Table 9a).

Table 9b shows that the G allele was the most common allele in both controls (75%) and heroin-dependent subjects (72.1%). Moreover, a slightly higher prevalence of the A allele was found in the heroin-dependent group (27.9% vs 25%) as compared with the control group. However, there was no significant difference in allele frequencies between the control and heroin-dependent group (p=0.46; Table 9b). A statistically significant difference between heroin-dependent subjects and controls was also not observed in the genotype frequency (p=0.07) as shown in Table 9a for the *Nci*I polymorphism of the GABRG2 gene.

CHAPTER FOUR DISCUSSION & CONCLUSION

In the present study, the involvement of polymorphisms at the hMOR, hDOR, DAT, DRD2 and GABRG2 genes in heroin dependence were examined. Polymorphisms that were investigated included the A118G polymorphism at exon 1, the C1031G polymorphism at intron 2 of the hMOR gene, the T921C polymorphism at exon 3 of the hDOR gene, the 3' VNTR polymorphism of the DAT gene, the *Taq*I A polymorphism of the DRD2 gene and the *Nci*I polymorphism of the GABRG2 gene. Statistical significance was found between the heroin-dependent and control subjects of the A118G polymorphism of the hMOR gene in both the genotype (p=0.046; see Table 2a) and allelic frequencies (p=0.016; see Table 2b). A significant difference was also found in the allelic frequency (p=0.014; see Table 3b) of the C1031G polymorphism of the hMOR gene as well as the genotype (p=0.038; see Table 4a) and the allelic frequencies (p=0.028; see Table 4b) of the T921C polymorphism of the hDOR gene. However, no statistical significance was found in the polymorphisms of the DAT, DRD2 and GABRG2 genes (see Tables 7-9).

Addiction Severity Index Data

Apart from investigating into associations of the different gene polymorphisms between the heroin-dependent and control subjects, ASI was also obtained from the heroin-dependent subjects to study various profiles that are related to addiction behaviour.

From the distribution of ASI – Medical status, it was shown that 151 (75.5%) subjects

scored a 0, meaning no medical problems exist and treatment is probably not necessary. Only 10% of the subjects scored a 1 and 14.5% of the subjects were distributed between 2-8 on the scale. Thus, the medical status of 85.5% of the heroin-dependent subjects studied was satisfactory. Therefore medical problem may not be the major factor that contributes to heroin dependence because they did not suffer from serious illness which requires the use of heroin to relieve pain. This suggestion is consistent with the result of the correlation among ASI factors. No statistical significance was found between medical and drug factors. Significant correlation was only found between medical and family/social relationships status ($p=0.004$, $r=0.192$; Table 1). From the distribution of the education standards of the heroin-dependent subjects, it was found that 96.3% of these subjects have an education level of no more than Form 3 standard. As a result, the majority of them work irregular hours each week and their jobs are often part-time in nature. From the distribution of the ASI – Employment indices, it can be seen that the majority of them were distributed at 4 to 8 on the scale, suggesting a problem with obtaining employment. This is probably related to their educational background, which resulted in a lack of marketable skills, thus contributing to them having employment problems. Moreover, Pearson's correlation showed that a statistical significance was found between employment and psychiatry ($p=5.63\times10^{-5}$, $r=0.274$; Table 1). This suggests that lack of employment may precipitate psychiatric symptoms like depression, anxiety, tension or even suicide attempts.

The ASI – Drug distribution showed that the majority (78.5%) of the subjects was distributed between 2 to 6 on the scale with 21.5% of subjects scoring 0 - 1 and 7 - 9.

This suggests that the majority of them have a drug problem. Since all of these subjects were diagnosed to be heroin-dependent based on the DSM IV diagnostic criteria and that the average age of drug use is 14 ± 9.1 years (Fig. 3), it is therefore not surprising that nearly 80% of subjects experienced drug-related problems. A significant correlation was found between drug and legal factor ($p=4.91x10^{-4}$, r=0.381; Table 1). This maybe due to the frequent police charges that these subjects received for possession of drugs or drug paraphernalia.

In the ASI – Family/social relationships section, the heroin-dependent subjects were asked about the nature of their relationships with their family members and friends. 37% of subjects showed they had no problems (score of 0) in getting along with their family and friends. 43% of them scored between 1 and 3 and 20% of subjects scored between 4 - 9 on the scale. Significant correlations were found between family/social relationships and medical (p=0.004, r=0.192; Table 1) status as well as family/social relationships and psychiatry status (p=3.76x10-7, r=0.465; Table 1). It is suggested that the medical and emotional problems experienced by the heroin-dependent subjects may result in poor inter-personal relationships, which may in turn result in conflicts with family members and friends.

The distribution of ASI - Psychiatry showed that 82% of the subjects scored between 0 to 3 on the scale. According to the ASI questions asked, the emotional problems they experienced like serious depression, anxiety, tension, problems in concentration and violent behaviour are mainly attributed to the dissatisfaction with employment, family/social relationships or the success in detoxification. Therefore, it is reasonable

to see there were significant correlations between psychiatry related problems and employment ($p=5.63x10^{-5}$, r=0.274; Table 1), family/social relationships (p=3.76x10-7, r=0.465, Table 1) and medical status (p=0.002, r=0.244; Table 1).

Thus, 3 (medical, employment and family/social relationships) out of 5 (medical, employment, drug, legal and family/social relationships) factors in the ASI were found to be correlated with psychiatric problems but not with drug problems. It seems therefore that environmental factors mainly affect the psychiatry status of the heroin-dependent subjects and not directly the nature of heroin dependence. For the heroin-dependent aspect, it has been suggested that genetic factor may play a major role in heroin dependence. It was been stated that in heroin abuse, a large unique contribution (38%) can be linked directly to be from genetic factors when compared to other drugs like marijuana, stimulant, sedative or psychedelic drugs (Tsuang et al., 1998). The present findings are in support of this tenet since ASI only showed a significant correlation between drug and legal status and not with other factors like psychiatry and medical.

In order to examine whether there is a correlation between the genetic and environment factors, the correlation between factors of ASI and allelic frequencies was also studied. Statistically significant correlations were found only between the C1031G polymorphism of the hMOR gene and family/social relationships status (p=0.00005) as well as psychiatric status (p=0.0068). Although the functional role of the C1031G polymorphism at intron 2 of hMOR gene is still unclear, it may be possible that this polymorphism or other nearby mutation site which is in linkage

disequilibrium with itself may play a role in the determination of personality or susceptibility to psychiatric disorders of these heroin-dependent subjects, which may in turn account for the poor relationships that they have with family members and in the society.

A118G Polymorphism in Exon 1 of the hMOR Gene

The biological aspect of heroin dependence has been investigated in recent years. It was hypothesized that reward is tonically activated by β-endorphin, an endogenous opioid that activates the MOR (Herz 1997). The functional significance of endogenous β-endorphin in reward also emerges from experimental results in rats with bilateral radiofrequency lesions of the mediobasal hypothalamus, whose hypothalamic β-endorphin content was largely abolished. Results showed a marked reduction in their display of aversive effects to naloxone. The attenuated aversive effects of the opioid antagonist are explained in terms of reduced β-endorphin release in limbic areas (Khachaturian et al., 1985; Mucha et al., 1985; Herz 1997).

The MOR is the primary action site for heroin and endogenous β-endorphin (Pasternak anf Wood 1986). The A118G variant of MOR binds β-endorphin approximately 3 times more tightly than the wild type MOR. Also, β-endorphin is approximately 3 times more potent at the A118G variant MOR when compared with the wild type receptor. These results indicated that the A118G polymorphism of MOR can alter binding and signal transduction in the receptor and may have implications for normal physiology, therapeutics and vulnerability to develop diverse diseases

including heroin addiction (Bond et al, 1998). These authors showed a significant difference in allele frequency for exon 1 of A118G ($p=0.028$) amongst the Hispanics, Africa-Americans and Caucasians irrespective of their opioid dependence status. However, it was noted that within the Hispanics but not in Africa-Americans or Caucasians, the A118G variant allele was present in a significantly higher proportion of non-opioid-dependent subjects when compared with the opioid-dependent subjects. This suggests that ethnic variance exists for these mutations. In view of this finding, we have attempted to screen DNA samples from heroin-dependent as well as control Chinese subjects for the A118G exon 1 polymorphism in order to establish whether such ethnic variance also exists in the Chinese population. The results in the present study showed that a significant association also exists for the A118G polymorphism in the hMOR gene in the Chinese heroin-dependent subjects. The findings showed that the A allele is more common than the G allele in both control and heroin-dependent subjects. However, more heroin-dependent subjects were shown to carry the G allele than the controls. The increase of G allele was due to the increase in GG and AG genotypes with a decrease in the number of AA genotype. With these findings, it could be concluded that the A118G polymorphism may be involve in heroin addiction. This study suggested that the A allele may have a protective role in the development of heroin dependence. With the increase in G allele and decrease in A allele in the heroin-dependent subjects, it may be concluded that subjects with the A118G variant of MOR, with 3-times higher affinity and potency for β-endorphin, may in turn make the individual more predisposed to heroin dependence. This suggested that the genetic susceptibility to heroin dependence may be related to an increased responsiveness of the endogenous opioid system to heroin. Therefore, the

difference in sensitivity to the rewarding effect of heroin may account for the different extent of vulnerability to heroin dependence.

On the contrary, Bond et al. (1998) suggested that the G allele might have a relative protective function in opioid dependence among the Hispanics since the A118G variant was present in a significantly (p=0.0041) higher proportion in the non-opioid-dependent Hispanic subjects. When the allelic frequency of the Chinese population in the present study was compared to the three different ethnic groups reported in Bond et al. (1998), the A allele frequency in both the control (70.6%) and heroin-dependent subjects (60.5%) in the Chinese subjects were lower to that reported in the Africa-American (98.4%), Caucasian (88.5%) and Hispanic (85.8%) subjects (Bond C et al., 1998). This suggests that the Chinese population is more predisposed to heroin dependence as compared with another three populations (see Appendix III). These authors claimed that this may be due to the differences in allelic frequencies in different populations since a significant difference of allele frequencies exist among Africa-Americans, Caucasians and Hispanics irrespective of opioid dependence (p=0.028) (Bond et al., 1998). However, the difference in allelic frequency of 10.1% for the A118G variant in the present population is similar to that reported in other studies (Berrettini et al., 1997; Bond et al., 1998). Therefore, this maybe the result of a linkage disequilibrium in the different populations being reversed with the causative mutation being nearby. That means a causative mutation may have arisen independently in different populations (Breen et al., 1999). As a result, one is near the A allele and another is near the G allele. Thus, G allele was found to be a predispose factor in the Chinese population while the A allele may be a risk factor in the Africa-

American, Caucasian or Hispanic populations.

In addition, the A118G variant predicts an Asn-to-Asp change in amino acid residue 40 of the receptor. This residue in the most common sequence of the MOR is a putative site for N-glycosylation (Mestek et al., 1995). Thus, the A118G variant would result in the loss of a putative N-glycosylation site. The position of amino acid 40 is in the N-terminal region of the MOR, which is predicted to be in the extracellular space (Chen et al., 1993). It remains to be determined whether there is any variant of the hMOR gene that can alter the tertiary structure of the MOR or change gene expression, which may account for the inter-individual differences in the predisposition to heroin dependence.

C1031G Polymorphism in Intron 2 of the hMOR Gene

A C1031G polymorphism in intron 2 of the hMOR gene was also investigated. It was chosen to see if other variants of the hMOR gene also confer a relevant effect on heroin dependence. A significant difference in allelic frequency was found between the heroin-dependent and control subjects. It seemed that the G allele might play a role in the predisposition of heroin dependence since there is a significant increase in allelic frequency for the G allele in the heroin-dependent subjects. However, no significant difference was found in the genotype frequency ($p=0.094$) although a trend can be observed, as it can be noted that the number of GG homozygotes in the heroin-dependent subjects was double that of the controls. The functional role of this polymorphism on heroin dependence, however, remains to be determined. Limited

studies revealed that morphine-6β-glucuronide (M6G) and heroin did not produce antinociceptive responses in MOR knockout mice generated by the deletion of exons 2 and 3 (Kitanaka et al., 1998; Loh et al., 1998) but retained antinociceptive activities in mice generated by an exon 1 deletion (Schuller et al., 1999). Significant levels of M6G binding and the presence of mRNAs detected with the exon 2 and 3 primers were demonstrated in mutant mice with a MOR exon 1 deletion. The observations suggest the exon 2 and 3 of the hMOR gene may be specific for the M6G pharmacological actions (Law et al., 2000).

Binding studies demonstrated that humans differ from one another in their MOR densities in the brain (Uhl et al., 1999). Furthermore, MOR densities have been shown to be higher in alcohol-preferring rats relative to their non-preferring counterparts in the NAc and VTA (de Waele et al., 1995; Soini et al., 1999). It was postulated that a link may exist between MOR binding potential and alcohol preference (Roberts et al., 2000). Thus, it is worth noting that the possibilities of the involvement of polymorphisms in exons 1 and 2 of hMOR gene in the expression of MOR, which in turn may affect the susceptibility to heroin dependence.

The combined genotypes of the A118G and the C1031G polymorphisms of the hMOR gene in the control and heroin-dependent subjects revealed that the vulnerability of heroin dependence is more robust when the individual carries a double dose (GG of A118G and GG of C1031G) of A118G and C1031G polymorphisms than in those who carry a zero or single dosage ($p=0.001$, RO=3.396; see Table 5). This further supports an association of the G allele with susceptibility to

heroin dependence. In addition, it was proposed that the hMOR is likely a genetic risk factor associated with heroin dependence.

T921C Polymorphism in Exon 3 of the hDOR Gene

Several studies have proposed that the hDOR gene may be critical in the development of morphine-induced tolerance and dependence (Abdelhamid et al., 1991; Suzuki et al., 1994; Fundytus et al., 1995; Kest et al., 1996). The present study revealed a significant difference in allelic variation in exon 3 of the hDOR gene between the heroin-dependent subjects and the controls. The T allele was more frequent than the C allele in both the control group and heroin-dependent groups. A higher percentage of the heroin-dependent subjects carried the C allele, due to an increase in CC and TC genotypes with a decrease in TT. The correlation between the genotype of the T921C polymorphism of the hDOR gene and heroin dependence suggested a causal contribution of genetic variation in the hDOR gene to the development of heroin addiction, although the mechanism is still unclear. The presence of the C allele was previously suggested to significantly increase the risk for heroin dependence in a German population when the C17T polymorphism was examined (Mayer et al., 1997). It can be suggested that CC homozygous carriers may be more predisposed to heroin dependence. On the other hand, TT homozygotes may play some sort of role in the prevention from addiction. This means that the C and T allele seems to favour and discourage heroin addiction respectively.

The mechanisms underlying the association between T921C polymorphism of the

hDOR gene and heroin dependence remain unknown. Since the silent T to C transition in the coding region did not alter the receptor protein sequence (Mayer et al., 1997), the T921C polymorphism may not be the functional variation that contributes to heroin dependence. However, it has been demonstrated that there is an upregulation of the DOR binding site accompanying the development of morphine tolerance and dependence in mice (Abdelhamid and Takemori, 1990). Therefore, it was speculated that either the reported polymorphism in exon 3 itself or another closely linked polymorphism within the gene could alter the expression of the hDOR gene. Gelernter and colleagues identified one novel variant in exon 1, T80G polymorphism of the hDOR gene, which predicts a change in amino acid sequence from phenylalanine (80T) to cysteine (80G) (Glernter and Kranzler, 2000). As a result, further experiments might reveal whether this variant would contribute to the previously reported association results involving a T921C polymorphism. In addition, a double dose (GG of A118G and CC of T921C) of A118G and T921C polymorphisms gives a robust susceptibility to heroin dependence (p=0.001, RO=3.434; Table 5). Furthermore, a more robust vulnerability to heroin dependence was found in subjects that carried a double dose (GG of C1031 and CC of T921C) of C1031G of hMOR and T921C of hDOR polymorphisms (p=0.001, RO=3.520; Table 5).

3' VNTR Polymorphism of DAT Gene

Previous studies have shown an interaction between the opioid system in the brain with that of the dopamine system (Kalivas and Duffy, 1990; Acquas et al., 1991;

Leone et al., 1991; Spanagel et al., 1992). Since opioids have the ability to increase dopamine release in the NAc, this action has been suggested to be related to their reinforcing properties (Van Ree et al., 1999). Moreover, animals will press a lever to receive injections of opioids directly into the VTA, wherein the cell bodies of the mesocorticolimbic dopaminergic system are located (Van Ree and De Wied, 1980; Bozarth and Wise, 1981b; Welzl et al., 1989) and such injection increase dopamine release in the NAc (Leone et al., 1991; Rada et al., 1991). DAT is the key element in controlling dopamine levels at the synapse. In the present study, the possibility of the contribution of the dopamine receptor and transporter genes to the vulnerability of heroin dependence was also investigated.

198 out of 297 (heroin-dependent subjects and controls) had the 10/10 genotype of the 3' VNTR of DAT gene (Table 7a). It can be seen that the 10 allele of DAT gene is the most common allele, this was also reported in other studies (Nakatome et al., 1995; Gelernter et al., 1998; Lerman et al., 1999). However, around 90% of our participants had at least one 10 allele, resulting in a very skewed distribution. Therefore analyses of 3' VNTR of DAT gene were based on the presence or absence of the next common allele, the 9 allele. The 9 allele was more frequent in heroin-dependent subjects than in controls. Nevertheless, there was no significant difference in genotype frequency ($p=0.519$) nor allelic frequency ($p=0.351$) for the association between the 3' VNTR polymorphism of DAT gene and heroin dependence.

The 9 allele has been shown to be present more frequently in cocaine abusers who reported paranoid ideation with cocaine use (Gelernter et al., 1994). A linkage

between the DAT gene and bipolar disorder has been reported (Kelsoe et al., 1996). In addition, opposite abnormalities of DAT density in violent and nonviolent alcoholics were observed (Tiihonen et al., 1995). This implies that differences in the expression of the genotype may account for the phenotypic variation in personality traits of addicts (Schmidt et al., 1998). In the present study, although no association between the 3' VNTR polymorphism of the DAT gene and heroin dependence were found, one cannot rule out that this gene may still exert some indirect influence on a subject's vulnerability to heroin dependence through a variation in personality traits.

Since the chronic treatment of rats with morphine significantly decreases the DAT density in NAc (Simantov 1993), it was believed that DAT gene may participated in the heroin dependence. However, the 9 allele is in the 3' untranslated region of the DAT gene. It is unlikely that a polymorphism in the 3' untranslated region will alter the gene expression or protein structure of DAT. Thus, it may be in linkage disequilibrium with a mutation close to it. Variable results in population-based association studies of 3' VNTR polymorphism of the DAT gene may partly be due to unappreciated population differences in the frequencies of alleles. For instance, the 7 allele of 3' VNTR polymorphism of the DAT gene is more frequent in alcoholics who are heterozygous for the inactive aldehyde dehydrogenase-2 allele (ALDH2*2) in the Japanese population (Muramatsu and Higuchi, 1995). However, only 1% of this polymorphism was found in our Chinese participants. Therefore, although a significant association between the 9 allele of the DAT gene and heroin dependence was not be found in the present study, it remains to be determined whether such an association may be found in other ethnic populations.

*Taq*I A Polymorphism of DRD2 Gene

DRD2, another candidate gene in the dopaminergic system, has been investigated extensively by many investigators. The A1 allele of the DRD2 gene has been correlated with increased risk of severe alcoholism (Blum et al., 1991; Amadeo et al.,1993; Arinami et al., 1993; Noble et al., 1994; Bau et al., 2000; Markianos et al., 2000), cocaine dependence (Noble et al., 1993; Comings et al., 1994), obesity (Comings et al., 1993), attention deficit hyperactivity disorder (Comings et al., 1991), Tourette syndrome (Comings et al., 1991; Devor, 1992), pathological gambling (Comings et al., 1994), smoking (George et al., 1993; Noble et al., 1994; Bierut et al., 2000), enhanced response to stress (Berman and Noble, 1997), novelty seeking and reward (Noble et al., 1998).

It was reported that the diminished CNS dopaminergic activity in A1 allele carriers as compared to A2 allele carriers was due to the reduced number of DRD2 (Noble et al., 1994; Berman and Noble, 1995) instead of the difference in the affinity of DRD2 between A1 and A2 carriers (Noble et al., 1991; Thompson et al., 1997). Therefore, it may be postulated that subjects inheriting the A1 allele of DRD2 gene may compensate for a deficit in their mesocorticolimbic dopaminergic pathways by the use of an agent known to increase brain dopamine levels such as heroin or alcohol (Van Ree et al., 1999). The subsequent stimulation by dopamine of A1 allelic carriers of DRD2 could be strongly reinforcing and the pleasurable and rewarding experience of substances of abuse subsequently may increase the risk for addiction. Thus, it was speculated that the A1 allele of DRD2 maybe the predisposing factor for substance

dependence, like heroin dependence.

However, in the present study, there was no significant difference in genotype (p=0.536) nor allelic frequency (p=0.711) for the association between the *Taq*I A polymorphism of DRD2 and heroin dependence between the heroin-dependent and control groups. Since the *Taq* I A polymorphism of DRD2 gene is located at the 3' non-coding region. It has been proposed that the most likely explanation for the association between A1 allele of the DRD2 gene and heroin dependence is that the sequence variation causing the *Taq*I A polymorphism is in linkage disequilibrium with functional allelic variants that lie at a distance, upstream or even downstream from the corresponding coding region (Amadeo et al., 1993). As the A1 allele of the DRD2 gene is associated with a number of disorders, it could reduce the transcriptional activity of the DRD2 gene and thus modify addictive behaviour like heroin dependence. As a result, it may be possible that the A1 allele of the DRD2 gene act as a modifying gene rather than as the primary etiological agent (Comings et al., 1991). As fewer than half of the subjects were found to carry the A1 allele, it cannot be with total conviction that one could state that the heterozygous composition is the cause of each disorder.

Recently, a functional polymorphism in the 5' promoter region of DRD2 gene has been identified (-141C INS/Del), which appears to affect susceptibility to schizophrenia (Arinami et al., 1997). Thus it encourages further investigation of the DRD2 gene to elucidate its role in governing heroin-dependent behaviour.

*Nci*I Polymorphism of GABRG2 Gene

In the present study, a significant association was not be found between the *Nci*I polymorphism of the GABRG2 gene and heroin dependence. This may be due to the presence of linkage disequilibrium with functional allelic variants in other regions of these gene, such as in the promoter, introns or exons. Although little is known about the specific role of the GABA A receptor in addictive behaviour (Loh et al., 1999), it was postulated that changes in subunit expression may alter the assembly of GABA A receptors which could account for the changes in receptor function and binding (Grobin et al., 1998). Subjects who carry the mutated GABA A receptor gene may have diminished activation of chloride channel, which in turn affect the intoxicating effects of the drug (Noble et al., 1998). Therefore, we have postulated that the activity of GABA-mediated chloride channel is reduced in the carriers with GABRG2 variant. Consequently, large amounts of heroin may have to be taken, resulting to an increasing risk in the development of heroin dependence. The proposed alternation in subunit expression may be an endogenous regulatory mechanism controlling the activity of GABA A receptors (Grobin et al., 1998). Further studies looking at other polymorphic sites at the GABA A receptor gene are welcome to reveal the involvement of this receptor in heroin dependence.

It can be concluded that the G allele of the A118G and C1031G polymorphisms of the hMOR gene are both associated with heroin dependence. Moreover, significant association were found between the C allele of T921C polymorphism of hDOR gene and heroin dependence in the Hong Kong Chinese population. However, so far there

is no statistical significant association between heroin dependence and those polymorphisms in DAT, DRD2 and GABRG2 genes that were examined in this study. It seems that opioid receptor genes such as both hMOR and hDOR genes may be genetic risk factors for heroin dependence. However, it is speculated that no single gene may be either necessary or sufficient to explain the likelihood of the development of addictive behaviour, for instance heroin dependence. It seems from the present study that a combination of genes and their interaction may be likely involved. It is suggested that the combined genotypes of these opioid receptor genes will make the vulnerability of heroin dependence more robust. Thus, each candidate gene contributes to a certain extent and the cumulative effects of their functional alteration produces a more robust influence. Based on these findings, further study on other nearby polymorphic sites is essential because the polymorphisms examined in this study may be in linkage disequilibrium with other potential candidate genes.

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APPENDIX I Addiction Severity Index

MEDICAL STATUS

* 1. How many times in your life have you been hospitalized for medical problems? *(Include o.d.'s, d.t.'s, exclude detox.)*

2. How long ago was your last hospitalization for a physical problem — YRS. — MOS.

3. Do you have any chronic medical problems which continue to interfere with your life?
0 - No
1 - Yes _____ Specify

* 4. Are you taking any prescribed medication on a regular basis for a physical problem?
0 - No 1 - Yes

5. Do you receive a pension for a physical disability? *(Exclude psychiatric disability.)*
0 - No
1 - Yes _____ Specify

6. How many days have you experienced medical problems in the past 30?

FOR QUESTIONS 7 & 8 PLEASE ASK PATIENT TO USE THE PATIENT'S RATING SCALE

7. How troubled or bothered have you been by these medical problems in the past 30 days?

Comments

8. How important to you now is treatment for these medical problems?

INTERVIEWER SEVERITY RATING

9. How would you rate the patient's need for medical treatment?

CONFIDENCE RATINGS

Is the above information significantly distorted by:

10. Patient's misrepresentation?
0 - No 1 - Yes

11. Patient's inability to understand?
0 - No 1 - Yes

EMPLOYMENT/SUPPORT STATUS

* 1. Education completed *(GED = 12 years)* — YRS. — MOS.

* 2. Training or technical education completed — MOS.

3. Do you have a profession, trade or skill?
0 - No
1 - Yes _____ Specify

4. Do you have a valid driver's license?
0 - No 1 - Yes

5. Do you have an automobile available for use? *(Answer "1" if no valid driver's license.)*
0 - No 1 - Yes

6. How long was your longest full-time job? — YRS. — MOS.

* 7. Usual (or last) occupation.
_____ *(Specify in detail)*

8. Does someone contribute to your support in any way?
0 - No 1 - Yes

9. (ONLY IF ITEM 8 IS YES) Does this constitute the majority of your support?
0 - No 1 - Yes

10. Usual employment pattern, past 3 years.
1 - full time (40 hrs/wk)
2 - part time (reg. hrs)
3 - part time (irreg., daywork)
4 - student
5 - service
6 - retired/disability
7 - unemployed
8 - in controlled environment

11. How many days were you paid for working in the past 30? (include "under the table" work.)

How much money did you receive from the following sources in the past 30 days?

12. Employment (net income)

13. Unemployment compensation

14. DPA

15. Pension, benefits or social security

16. Mate, family or friends (Money for personal expenses).

17. Illegal

Comments

18. How many people depend on you for the majority of their food, shelter, etc.?

19. How many days have you experienced employment problems in the past 30?

FOR QUESTIONS 20 & 21 PLEASE ASK PATIENT TO USE THE PATIENT'S RATING SCALE

20. How troubled or bothered have you been by these employment problems in the past 30 days?

21. How important to you *now* is counseling for these employment problems?

INTERVIEWER SEVERITY RATING

22. How would you rate the patient's need for employment counseling?

CONFIDENCE RATINGS

Is the above information significantly distorted by:

23. Patient's misrepresentation?
0 - No 1 - Yes

24. Patient's inability to understand?
0 - No 1 - Yes

DRUG/ALCOHOL USE

	PAST 30 Days	LIFETIME USE Yrs.	Rt of adm.
(01) Alcohol - Any use at all			
(02) Alcohol - To intoxication			
(03) Heroin			
(04) Methadone			
(05) Other opiates/ analgesics			
(06) Barbiturates			
(07) Other sed/ hyp/tranq.			
(08) Cocaine			
(09) Amphetamines			
(10) Cannabis			
(11) Hallucinogens			
(12) Inhalants			
(13) More than one substance per day (Incl. alcohol).			

Note: See manual for representative examples for each drug class

* Route of Administration: 1 = Oral, 2 = Nasal 3 = Smoking, 4 = Non IV inj., 5 = IV inj.

(14) Which substance is the major problem? *Please code as above or 00-No problem; 15-Alcohol & Drug (Dual addiction); 16-Polydrug; when not clear, ask patient.*

15. How long was your last period of voluntary abstinence from this major substance? *(00 - never abstinent)* ____ MOS.

16. How many months ago did this abstinence end? *(00 - still abstinent)*

* (17) How many times have you:
- Had alcohol d.t.'s
- Overdosed on drugs

* (18) How many times in your life have you been treated for:
- Alcohol Abuse:
- Drug Abuse:

* (19) How many of these were detox only?
- Alcohol
- Drug

(20) How much would you say you spent during the past 30 days on:
- Alcohol
- Drugs

Comments

(21) How many days have you been treated in an outpatient setting for alcohol or drugs in the past 30 days *(Include NA, AA).*

(22) How many days in the past 30 have you experienced:
- Alcohol Problems
- Drug Problems

FOR QUESTIONS 23 & 24 PLEASE ASK PATIENT TO USE THE PATIENT'S RATING SCALE

(23) How troubled or bothered have you been in the past 30 days by these:
- Alcohol Problems
- Drug Problems

(24) How important to you now is treatment for these:
- Alcohol Problems
- Drug Problems

INTERVIEWER SEVERITY RATING

(25) How would you rate the patient's need for treatment for:
- Alcohol Abuse
- Drug Abuse

CONFIDENCE RATINGS

Is the above information significantly distorted by:

(26) Patient's misrepresentation? 0 - No 1 - Yes

(27) Patient's inability to understand? 0 - No 1 - Yes

LEGAL STATUS

1. Was this admission prompted or suggested by the criminal justice system (judge, probation/ parole officer, etc.)

0 - No 1 - Yes

(2) Are you on probation or parole?

0 - No 1 - Yes

How many times in your life have you been arrested and charged with the following:

- *(01) - shoplifting/vandalism
- *(02) - parole/probation violations
- *(03) - drug charges
- *(06) - forgery
- *(07) - weapons offense
- *(08) - burglary, larceny, B & E
- *(09) - robbery
- *(10) - assault
- *(11) - arson
- *(12) - rape
- *(13) - homicide, manslaughter
- *(14A) - prostitution
- *(14B) - contempt of court
- *(14C) - other

*(15) How many of these charges resulted in convictions?

How many times in your life have you been charged with the following:

- *(16) Disorderly conduct, vagrancy, public intoxication
- *(17) Driving while intoxicated
- *(18) Major driving violations (reckless driving, speeding, no license, etc.)

*(19) How many months were you incarcerated in your life? MOS.

20. How long was your last incarceration? MOS.

21. What was it for? *(Use code 3-14, 16-18, if multiple charges, code most severe)*

(22) Are you presently awaiting charges, trial or sentence?
0 - No 1 - Yes

(23) What for *(If multiple charges, use most severe)*.

(24) How many days in the past 30 were you detained or incarcerated?

Comments

(25) How many days in the past 30 have you engaged in illegal activities for profit?

FOR QUESTIONS 26 & 27 PLEASE ASK PATIENT TO USE THE PATIENT'S RATING SCALE

(26) How serious do you feel your present legal problems are? *(Exclude civil problems)*

(27) How important to you *now* is counselling or referral for these legal problems?

INTERVIEWER SEVERITY RATING

(28) How would you rate the patient's need for legal services or counselling?

CONFIDENCE RATINGS

Is the above information significantly distorted by:

(29) Patient's misrepresentation?
0 - No 1 - Yes

(30) Patient's inability to understand?
0 - No 1 - Yes

FAMILY HISTORY

Have any of your relatives had what you would call a significant drinking, drug use or psych problem- one that did or should have led to treatment?

Mother's Side	Alc	Drug	Psych
Grandmother			
Grandfather			
Mother			
Aunt			
Uncle			

Father's Side	Alc	Drug	Psych
Grandmother			
Grandfather			
Father			
Aunt			
Uncle			

Siblings	Alc	Drug	Psych
Brother #1			
Brother #2			
Sister #1			
Sister #2			

Direction: Place "0" in relative category where the answer is clearly no for all relatives in the category; "1" where the answer is clearly yes for any relative within the category "X" where the answer is uncertain or "I don't know" and "N" where there never was a relative from that category. Code most problematic relative in cases of multiple members per category.

FAMILY/SOCIAL RELATIONSHIPS

(1) Marital Status

1 - Married
2 - Remarried
3 - Widowed
4 - Separated
5 - Divorced
6 - Never Married

2. How long have you been in this marital status? YRS. MOS.
(If never married, since age 18).

(3) Are you satisfied with this situation?
0 - No
1 - Indifferent
2 - Yes

* (4) Usual living arrangements (past 3 yr.)
1 - With sexual partner and children
2 - With sexual partner alone
3 - With children alone
4 - With parents
5 - With family
6 - With friends
7 - Alone
8 - Controlled environment
9 - No stable arrangements

5. How long have you lived in these arrangements. YRS. MOS.
(If with parents or family, since age 18).

(6) Are you satisfied with these living arrangements?
0 - No
1 - Indifferent
2 - Yes

Do you live with anyone who:
0 = No 1 = Yes

6A. Has a current alcohol problem?

6B. Uses non-prescribed drugs?

(7) With whom do you spend most of your free time:
1 - Family
2 - Friends
3 - Alone

(8) Are you satisfied with spending your free time this way?
0 - No 1 - Indifferent 2 - Yes

(9) How many close friends do you have?

Direction for 9A-18: Place "0" in relative category where the answer is clearly no for all relatives in the category "1" where the answer is clearly yes for any relative within the category; "X" where the answer is uncertain or "I don't know" and "N" where there never was a relative from that category.

9A. Would you say you have had close, long lasting, personal relationships with any of the following people in your life:

Mother
Father
Brothers/Sisters
Sexual Partner/Spouse
Children
Friends

Have you had significant periods in which you have experienced serious problems getting along with:

0 - No 1 - Yes	PAST 30 DAYS	IN YOUR LIFE
(10) Mother		
(11) Father		
(12) Brothers/Sisters		
(13) Sexual partner/spouse		
(14) Children		
(15) Other significant family_____		
(16) Close friends		
(17) Neighbors		
(18) Co-Workers		

Did any of these people (10-18) abuse you: 0 =No; 1 = Yes

18A. Emotionally (make you feel bad through harsh words)?		
18B. Physically (cause you physical harm)?		
18C. Sexually (force sexual advances or sexual acts)?		

(19) How many days in the past 30 have you had serious conflicts:
A with your family?
B with other people? (excluding family)

FOR QUESTIONS 20-23 PLEASE ASK PATIENT TO USE THE PATIENT'S RATING SCALE

How troubled or bothered have you been in the past 30 days by these:

(20) Family problems
(21) Social problems

How important to you now is treatment or counseling for these:

(22) Family problems
(23) Social problems

INTERVIEWER SEVERITY RATING

(24) How would you rate the patient's need for family and/or social counseling?

CONFIDENCE RATINGS

Is the above information significantly distorted by:

(25) Patient's misrepresentation?
0 - No 1 - Yes

(26) Patient's inability to understand?
0 - No 1 - Yes

Comments

PSYCHIATRIC STATUS

* (1) How many times have you been treated for any psychological or emotional problems?

In a hospital
As an Opt. or Priv. patient

2. Do you receive a pension for a psychiatric disability?
0 - No 1 - Yes

Have you had a significant period, (that was not a direct result of drug/alcohol use), in which you have:

0 - No 1 - Yes	PAST 30 DAYS	IN YOUR LIFE
(2) Experienced serious depression		
(4) Experienced serious anxiety or tension		
(5) Experienced hallucinations		
(6) Experienced trouble understanding, concentrating or remembering		
(7) Experienced trouble controlling violent behavior		
(8) Experienced serious thoughts of suicide		
(9) Attempted suicide		
(10) Been prescribed medication for any psychological/emotional problem		

(11) How many days in the past 30 have you experienced these psychological or emotional problems?

FOR QUESTIONS 12 & 13 PLEASE ASK PATIENT TO USE THE PATIENT'S RATING SCALE

(12) How much have you been troubled or bothered by these psychological or emotional problems in the past 30 days?

(13) How important to you now is treatment for these psychological problems?

THE FOLLOWING ITEMS ARE TO BE COMPLETED BY THE INTERVIEWER

At the time of the interview, is patient:
0 - No 1 - Yes

(14) Obviously depressed/withdrawn
(15) Obviously hostile
(16) Obviously anxious/nervous
(17) Having trouble with reality testing thought disorders, paranoid thinking
(18) Having trouble comprehending, concentrating, remembering.
(19) Having suicidal thoughts

Comments

INTERVIEWER SEVERITY RATING

(20) How would you rate the patient's need for psychiatric/psychological treatment?

CONFIDENCE RATINGS

Is the above information significantly distorted by:

(21) Patient's misrepresentation?
0 - No 1 - Yes

(22) Patient's inability to understand?
0 - No 1 - Yes

APPENDIX II Table of Severity Ratings

Client Rating	Interviewewer Severity Rating Range	Final Severity Rating
0	0	0
0	0 1 2	0
0	1 2 3	1
0	2 3 4	2
0	3 4 5	3
0	4 5 6	4
0	5 6 7	5
0	6 7 8	6
0	7 8 9	7
1	0	0
1	0 1 2	0
1	1 2 3	1
1	2 3 4	2
1	3 4 5	3
1	4 5 6	4
1	5 6 7	5
1	6 7 8	6
1	7 8 9	7
2	0	0
2	0 1 2	1
2	1 2 3	2
2	2 3 4	3
2	3 4 5	4
2	4 5 6	5
2	5 6 7	6
2	6 7 8	7
2	7 8 9	8
3	0	0
3	0 1 2	2
3	1 2 3	3
3	2 3 4	4
3	3 4 5	5
3	4 5 6	6
3	5 6 7	7
3	6 7 8	8
3	7 8 9	9
4	0	0
4	0 1 2	2
4	1 2 3	3
4	2 3 4	4
4	3 4 5	5
4	4 5 6	6
4	5 6 7	7
4	6 7 8	8
4	7 8 9	9

APPENDIX III

	A allele	G allele	Total
Hispanic	85.5%	14.2%	134
African-American	98.4%	1.6%	62
Caucasian	88.5%	11.5%	104
Chinese	63.8%	36.2%	297

APPENDIX IV

Single Nucleotide Polymorphism	Nucleotide position	Corresponding amino acid change
hMOR A118G	118	Asn → Asp
hMOR C1031G	1031	Ser → Cys
hDOR T921C	921	Gly → Gly